

Date: 2/28/02 Express Mail Label No. EV 05202856545

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5 Attorney's Docket No.: 3298.1003-000

USE OF METABOLIC PHENOTYPING
IN INDIVIDUALIZED TREATMENT WITH AMONAFIDE

RELATED APPLICATION

10 This application is a new application which claims
the benefit of U.S. Provisional Application No.
60/271,714, filed on February 28, 2001. The entire
teachings of the above application is incorporated herein
by reference.

15

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the individualization of
therapy. More specifically, the present invention relates
20 to the use of metabolic phenotyping in individualized
treatment with the drug amonafide.

(b) Description of the Prior Art

Description of the Prior Art

For the majority of drugs (or xenobiotics)
25 administered to humans, their fate is to be metabolized in
the liver, into a form less toxic and lipophilic with
their subsequent excretion in the urine. Their metabolism

involves two systems (Phase I and Phase II) which act consecutively: Phase I enzymes include the cytochrome P450 system which includes at least 20 enzymes catalyzing oxidation reactions as well as carboxylesterase, amindases, epoxide hydrolase, quinine reductase, alcohol and aldehyde dehydrogenase, xanthine oxidase and flavin-containing monooxygenase. These enzymes are localized in the microsomal fraction. Phase II enzymes include the conjugation system which involves at least 5 enzymes including, N-acetyltransferases (NAT), UDP-glucuronyltransferases (UGT), sulfotransferases (SUT), and glutathione-S-transferases (GST). A detailed description of the complex human drug metabolizing systems is provided in Kumar and Surapaneni (Medicinal Res. Rev. (2001) 21(5):397-411) and patent application WO 01/59127 A2.

The metabolism of a drug and its movement through the body (pharmacokinetics) are important in determining its effects, toxicity, and interactions with other drugs. The three processes governing pharmacokinetics are the absorption of the drug, distribution to various tissues, and elimination of drug metabolites. These processes are intimately coupled to drug metabolism, since a variety of metabolic modifications alter most of the physicochemical and pharmacological properties of drugs, including solubility, binding to receptors, and excretion rates. The metabolic pathways which modify drugs also accept a variety of naturally occurring substrates such as steroids, fatty acids, prostaglandins, leukotrienes, and vitamins. The enzymes in these pathways are therefore important sites of biochemical and pharmacological

interaction between natural compounds, drugs, carcinogens, mutagens, and xenobiotics.

It has long been appreciated that inherited differences in drug metabolism lead to drastically different levels of drug efficacy and toxicity among individuals. For drugs with narrow therapeutic indices, or drugs which require bioactivation (such as codeine), these polymorphisms can be critical. Moreover, promising new drugs are frequently eliminated in clinical trials based on toxicities which may only affect a segment of the individuals in a target group. Advances in pharmacogenomics research, of which drug metabolizing enzymes constitute an important part, are promising to expand the tools and information that can be brought to bear on questions of drug efficacy and toxicity (See Evans, W. E. and R. V. Relling (1999) Science 286: 487-491).

Drug metabolic reactions are categorized as Phase I, which functionalize the drug molecule and prepare it for further metabolism, and Phase II, which are conjugative. In general, Phase I reaction products are partially or fully inactive, and Phase II reaction products are the chief excreted species. However, Phase I reaction products are sometimes more active than the original administered drugs; this metabolic activation principle is exploited by pro-drugs (e. g. L-dopa). Additionally, some nontoxic compounds (e. g. aflatoxin, benzo [a] pyrene) are metabolized to toxic intermediates through these pathways. Phase I reactions are usually rate-limiting in drug metabolism. Prior exposure to the

compound, or other compounds, can induce the expression of Phase I enzymes however, and thereby increase substrate flux through the metabolic pathways. (See Klassen, C. D., Amdur, M. O. and J. Doull (1996) Casarett and Doull's Toxicology : The Basic Science of Poisons, McGraw-Hill, New York, NY, pp. 113-186; Katzung, B. G. (1995) Basic and Clinical Pharmacology, Appleton and Lange, Norwalk, CT, pp. 48-59; Gibson, G. G. and Skett, P. (1994) Introduction to Drug Metabolism, Blackie Academic and Professional, London.)

Drug metabolizing enzymes (DMEs) have broad substrate specificities. This can be contrasted to the immune system, where a large and diverse population of antibodies is highly specific for their antigens. The ability of DMEs to metabolize a wide variety of molecules creates the potential for drug interactions at the level of metabolism. For example, the induction of a DME by one compound may affect the metabolism of another compound by the enzyme.

DMEs have been classified according to the type of reaction they catalyze and the cofactors involved. The major classes of Phase I enzymes include, but are not limited to, cytochrome P450 and flavin-containing monooxygenase. Other enzyme classes involved in Phase 1-type catalytic cycles and reactions include, but are not limited to, NADPH cytochrome P450 reductase (CPR), the microsomal cytochrome b5/NADH cytochrome b5 reductase system, the ferredoxin/ferredoxin reductase redox pair, aldo/keto reductases, and alcohol dehydrogenases. The major classes of Phase II enzymes include, but are not

limited to, UDP glucuronyltransferase, sulfotransferase, glutathione S-transferase, N-acyltransferase, and N-acetyl transferase.

5 **Cytochrome P450 and P450 catalytic cycle-associated enzymes**

Members of the cytochrome P450 superfamily of enzymes catalyze the oxidative metabolism of a variety of substrates, including natural compounds such as steroids, fatty acids, prostaglandins, leukotrienes, and vitamins, as well as drugs, carcinogens, mutagens, and xenobiotics. Cytochromes P450, also known as P450 heme-thiolate proteins, usually act as terminal oxidases in multi-component electron transfer chains, called P450-containing monooxygenase systems. Specific reactions catalyzed include hydroxylation, epoxidation, N-oxidation, sulfoxidation, N-, S-, and dealkylations, desulfation, deamination, and reduction of azo, nitro, and N-oxide groups. These reactions are involved in steroidogenesis of glucocorticoids, cortisols, estrogens, and androgens in animals; insecticide resistance in insects; herbicide resistance and flower coloring in plants; and environmental bioremediation by microorganisms. Cytochrome P450 actions on drugs, carcinogens, mutagens, and xenobiotics can result in detoxification or in conversion of the substance to a more toxic product. Cytochromes P450 are abundant in the liver, but also occur in other tissues; the enzymes are located in microsomes. (Graham-Lorence, S. and Peterson, J. A. (1996) FASEB J. 10: 206-214.)

Four hundred cytochromes P450 have been identified in diverse organisms including bacteria, fungi, plants, and animals (Graham-Lorence, supra). The B-class is found in prokaryotes and fungi, while the E-class is found in
5 bacteria, plants, insects, vertebrates, and mammals. Five subclasses or groups are found within the larger family of E-class cytochromes P450.

All cytochromes P450 use a heme cofactor and share structural attributes. Most cytochromes
10 P450 are 400 to 530 amino acids in length. The secondary structure of the enzyme is about 70% alpha-helical and about 22% beta-sheet. The region around the heme-binding site in the C-terminal part of the protein is conserved among cytochromes P450. A ten amino acid signature
15 sequence in this heme-iron ligand region has been identified which includes a conserved cysteine involved in binding the heme iron in the fifth coordination site. In eukaryotic cytochromes P450, a membrane-spanning region is usually found in the first 15-20 amino acids of the
20 protein, generally consisting of approximately 15 hydrophobic residues followed by a positively charged residue (Graham-Lorence, supra.).

Cytochrome P450 enzymes are involved in cell proliferation and development. The enzymes have roles in
25 chemical mutagenesis and carcinogenesis by metabolizing chemicals to reactive intermediates that form adducts with DNA (Nebert, D. W. and Gonzalez, F. J. (1987) Ann. Rev. Biochem. 56: 945-993). These adducts can cause nucleotide changes and DNA rearrangements that lead to oncogenesis.
30 Cytochrome P450 expression in liver and other tissues is

induced by xenobiotics such as polycyclic aromatic hydrocarbons, peroxisomal proliferators, phenobarbital, and the glucocorticoid dexamethasone (Dogra, S. C. et al. (1998) Clin. Exp. Pharmacol. Physiol. 25: 1-9). A
5 cytochrome P450 protein may participate in eye development as mutations in the P450 gene CYP1B1 cause primary congenital glaucoma.

Cytochromes P450 are associated with inflammation and infection. Hepatic cytochrome P450 activities are
10 profoundly affected by various infections and inflammatory stimuli, some of which are suppressed and some induced (Morgan, E. T. (1997) Drug Metab. Rev. 29: 1129-1188). Effects observed *in vivo* can be mimicked by proinflammatory cytokines and interferons. Autoantibodies
15 to two cytochrome P450 proteins were found in individuals with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a polyglandular autoimmune syndrome.

Mutations in cytochromes P450 have been linked to metabolic disorders, including congenital adrenal
20 hyperplasia, the most common adrenal disorder of infancy and childhood; pseudovitamin deficiency rickets; cerebrotendinous xanthomatosis, a lipid storage disease characterized by progressive neurologic dysfunction, premature atherosclerosis, and cataracts; and an inherited
25 resistance to the anticoagulant drugs coumarin and warfarin (Isselbacher, K. J. et al. (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, Inc. New York, NY, pp. 1968-1970; Takeyama, K. et al. (1997) Science 277: 1827-1830; Kitanaka, S. et al. (1998) N.
30 Engl. J. Med. 338: 653-661). Extremely high levels of

expression of the cytochrome P450 protein aromatase were found in a fibrolamellar hepatocellular carcinoma from a boy with severe gynecomastia (feminization) (Agarwal, V. R. (1998) J. Clin. Endocrinol. Metab. 83: 1797-1800).

5 The cytochrome P450 catalytic cycle is completed through reduction of cytochrome P450 by NADPH cytochrome P450 reductase (CPR). Another microsomal electron transport system consisting of cytochrome b5 and NADPH cytochrome b5 reductase has been widely viewed as a
10 minor contributor of electrons to the cytochrome P450 catalytic cycle. However, a recent report by Lamb, D. C. et al. (1999 ; FEBS Lett. 462: 283-8) identifies a *Candida albicans* cytochrome P450 (CYP51) which can be efficiently reduced and supported by the microsomal cytochrome
15 b5/NADPH cytochrome b5 reductase system. Therefore, there are likely many cytochromes P450 which are supported by this alternative electron donor system.

Cytochrome b5 reductase is also responsible for the reduction of oxidized hemoglobin (methemoglobin, or ferrihemoglobin, which is unable to carry oxygen) to the
20 active hemoglobin (ferrohemoglobin) in red blood cells. Methemoglobinemia results when there is a high level of oxidant drugs or an abnormal hemoglobin (hemoglobin M) which is not efficiently reduced. Methemoglobinemia can
25 also result from a hereditary deficiency in red cell cytochrome b5 reductase (Reviewed in Mansour, A. and Lurie, A. A. (1993) Am. J. Hematol. 42: 7-12).

Members of the cytochrome P450 family are also closely associated with vitamin D synthesis and
30 catabolism. Vitamin D exists as two biologically

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equivalent prohormones, ergocalciferol (vitamin D₂), produced in plant tissues and cholecalciferol (vitamin D₃), produced in animal tissues. The latter form, cholecalciferol, is formed upon the exposure of 7-dehydrocholesterol to near ultraviolet light (i.e., 290-310 nm), normally resulting from even minimal periods of skin exposure to sunlight (reviewed in Miller, W. L. and Portale, A. A. (2000) Trends in Endocrinology and Metabolism 11: 315-319).

Both prohormone forms are further metabolized in the liver to 25-hydroxyvitamin D (25(OH)D) by the enzyme 25-hydroxylase. 25(OH)D is the most abundant precursor form of vitamin D which must be further metabolized in the kidney to the active form, 1 α ,25-dihydroxyvitamin D (1 α ,25(OH)₂D), by the enzyme 25-hydroxyvitamin D 1 α -hydroxylase (1 α -hydroxylase). Regulation of 1 α ,25(OH)₂D production is primarily at this final step in the synthetic pathway. The activity of 1 α -hydroxylase depends upon several physiological factors including the circulating level of the enzyme product (1 α ,25(OH)₂D) and the levels of parathyroid hormone (PTH), calcitonin, insulin, calcium, phosphorus, growth hormone, and prolactin. Furthermore, extrarenal 1 α -hydroxylase activity has been reported, suggesting that tissue-specific, local regulation of 1 α ,25(OH)₂D production may also be biologically important. The catalysis of 1 α ,25(OH)₂D to 24,25-dihydroxyvitamin D (24,25(OH)₂D), involving the enzyme 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase), also occurs in the kidney. 24-hydroxylase

can also use $25(\text{OH})_2\text{D}$ as a substrate (Shinki, T. et al. (1997) Proc. Natl. Acad. Sci. U. S. A. 94: 12920-12925 ; Miller, W. L. and Portale, A. A. supra; and references within).

5 Vitamin D 25-hydroxylase, 1α -hydroxylase, and 24-hydroxylase are all NADPH-dependent, type I (mitochondrial) cytochrome P450 enzymes that show a high degree of homology with other members of the family. Vitamin D 25-hydroxylase also shows a broad substrate
10 specificity and may also perform 26-hydroxylation of bile acid intermediates and 25,26, and 27-hydroxylation of cholesterol (Dilworth, F. J. et al. (1995) J. Biol. Chem. 270: 16766-16774; Miller, W. L. and Portale, A. A. supra; and references within).

15 The active form of vitamin D ($1\alpha,25(\text{OH})_2\text{D}$) is involved in calcium and phosphate homeostasis and promotes the differentiation of myeloid and skin cells. Vitamin D deficiency resulting from deficiencies in the enzymes involved in vitamin D metabolism (e.g., 1α -hydroxylase)
20 causes hypocalcemia, hypophosphatemia, and vitamin D-dependent (sensitive) rickets, a disease characterized by loss of bone density and distinctive clinical features, including bandy or bow leggedness accompanied by a waddling gait. Deficiencies in vitamin D 25-hydroxylase
25 cause cerebrotendinous xanthomatosis, a lipid-storage disease characterized by the deposition of cholesterol and cholestanol in the Achilles' tendons, brain, lungs, and many other tissues. The disease presents with progressive neurologic dysfunction, including postpubescent cerebellar

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ataxia, atherosclerosis, and cataracts. Vitamin D 25-hydroxylase deficiency does not result in rickets, suggesting the existence of alternative pathways for the synthesis of 25 (OH) D (Griffin, J. E. and Zerwekh, J. E. (1983) J. Clin. Invest. 72: 1190-1199; Gamblin, G. T. et al. (1985) J. Clin. Invest. 75: 954-960; and W. L. and Portale, A. A. supra).

Ferredoxin and ferredoxin reductase are electron transport accessory proteins which support at least one human cytochrome P450 species, cytochrome P450c27 encoded by the CYP27 gene (Dilworth, F. J. et al. (1996) Biochem. J. 320: 267-71). A *Streptomyces sriseus* cytochrome P450, CYP104D1, was heterologously expressed in *E. coli* and found to be reduced by the endogenous ferredoxin and ferredoxin reductase enzymes (Taylor, M. et al. (1999) Biochem. Biophys. Res. Commun. 263: 838-42), suggesting that many cytochrome P450 species may be supported by the ferredoxin/ferredoxin reductase pair. Ferredoxin reductase has also been found in a model drug metabolism system to reduce actinomycin D, an antitumor antibiotic, to a reactive free radical species (Flitter, W. D. and Mason, R. P. (1988) Arch. Biochem. Biophys. 267: 632-9).

Flavin-containing monooxygenase (FMO)

Flavin-containing monooxygenases (FMO) oxidize the nucleophilic nitrogen, sulfur, and phosphorus heteroatom of an exceptional range of substrates. Like cytochromes P450, FMOs are microsomal and use NADPH and O₂; there is also a great deal of substrate overlap with cytochromes

P450. The tissue distribution of FMOs includes liver, kidney, and lung.

There are five different known isoforms of FMO in mammals (FMO1, FMO2, FMO3, FMO4, and FMOS), which are expressed in a tissue-specific manner. The isoforms differ in their substrate specificities and other properties such as inhibition by various compounds and stereospecificity of reaction. FMOs have a 13 amino acid signature sequence, the components of which span the N-terminal two-thirds of the sequences and include the FAD binding region and the FATGY motif which has been found in many N-hydroxylating enzymes (Stehr, M. et al. (1998) Trends Biochem. Sci. 23: 56-57).

Specific reactions include oxidation of nucleophilic tertiary amines to N-oxides, secondary amines to hydroxylamines and nitrones, primary amines to hydroxylamines and oximes, and sulfur containing compounds and phosphines to S-and P-oxides. Hydrazines, iodides, selenides, and boroncontaining compounds are also substrates. Although FMOs appear similar to cytochromes P450 in their chemistry, they can generally be distinguished from cytochromes P450 *in vitro* based on, for example, the higher heat lability of FMOs and the nonionic detergent sensitivity of cytochromes P450; however, use of these properties in identification is complicated by further variation among FMO isoforms with respect to thermal stability and detergent sensitivity.

FMOs play important roles in the metabolism of several drugs and xenobiotics. FMO (FMO3 in liver) is predominantly responsible for metabolizing (S)-nicotine to

(S)-nicotine N-1'-oxide, which is excreted in urine. FMO is also involved in S-oxygenation of cimetidine, an H₂-antagonist widely used for the treatment of gastric ulcers. Liver-expressed forms of FMO are not under the same regulatory control as cytochrome P450. In rats, for example, phenobarbital treatment leads to the induction of cytochrome P450, but the repression of FMO1.

Endogenous substrates of FMO include cysteamine, which is oxidized to the disulfide, cystamin, and trimethylamine (TMA), which is metabolized to trimethylamine N-oxide. TMA smells like rotting fish, and mutations in the FMO3 isoform lead to large amounts of the malodorous free amine being excreted in sweat, urine, and breath. These symptoms have led to the designation fish-odor syndrome.

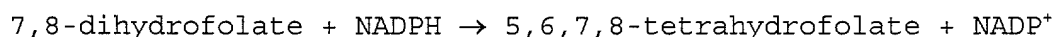
Lysyl Oxidase

Lysyl oxidase (lysine 6-oxidase, LO) is a copper-dependent amine oxidase involved in the formation of connective tissue matrices by crosslinking collagen and elastin. LO is secreted as a N-glycosylated precursor protein of approximately 50 kDa and cleaved to the mature form of the enzyme by a metalloprotease, although the precursor form is also active. The copper atom in LO is involved in the transport of electron to and from oxygen to facilitate the oxidative deamination of lysine residues in these extracellular matrix proteins. While the coordination of copper is essential to LO activity,

insufficient dietary intake of copper does not influence the expression of the apoenzyme. However, the absence of the functional LO is linked to the skeletal and vascular tissue disorders that are associated with dietary copper deficiency. LO is also inhibited by a variety of semicarbazides, hydrazines, and amino nitrites, as well as heparin. Beta-aminopropionitrile is a commonly used inhibitor. LO activity is increased in response to ozone, cadmium, and elevated levels of hormones released in response to local tissue trauma, such as transforming growth factor-beta, platelet-derived growth factor, angiotensin II, and fibroblast growth factor. Abnormalities in LO activity has been linked to Menkes syndrome and occipital horn syndrome. Cytosolic forms of the enzyme have been implicated in abnormal cell proliferation (reviewed in Rucker, R. B. et al. (1998) Am. J. Clin. Nutr. 67: 996S-1002S and Smith-Mungo. L. I. and Kagan, H. M. (1998) Matrix Biol. 16: 387-398).

Dihydrofolate Reductases

Dihydrofolate reductases (DHFR) are ubiquitous enzymes that catalyze the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, an essential step in the *de novo* synthesis of glycine and purines as well as the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). The basic reaction is as follows:



The enzymes can be inhibited by a number of dihydrofolate analogs, including trimethoprim and methotrexate. Since an abundance of TMP is required for DNA synthesis, rapidly dividing cells require the activity of DHFR. The replication of DNA viruses (i.e., herpesvirus) also requires high levels of DHFR activity. As a result, drugs that target DHFR have been used for cancer chemotherapy and to inhibit DNA virus replication. (For similar reasons, thymidylate synthetases are also target enzymes.) Drugs that inhibit DHFR are preferentially cytotoxic for rapidly dividing cells (or DNA virus-infected cells) but have no specificity, resulting in the indiscriminate destruction of dividing cells. Furthermore, cancer cells may become resistant to drugs such as methotrexate as a result of acquired transport defects or the duplication of one or more DHFR genes (Stryer, L (1988) Biochemistry. W. H Freeman and Co., Inc. New York. pp. 511-5619).

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Aldo/keto Reductases

Aldo/keto reductases are monomeric NADPH-dependent oxidoreductases with broad substrate specificities (Bohren, K. M. et al. (1989) J. Biol. Chem. 264: 9547-51). These enzymes catalyze the reduction of carbonyl-containing compounds, including carbonyl-containing sugars and aromatic compounds, to the corresponding alcohols. Therefore, a variety of carbonyl-containing drugs and xenobiotics are likely metabolized by enzymes of this class.

30

One known reaction catalyzed by a family member, aldose reductase, is the reduction of glucose to sorbitol, which is then further metabolized to fructose by sorbitol dehydrogenase. Under normal conditions, the reduction of glucose to sorbitol is a minor pathway. In hyperglycemic states, however, the accumulation of sorbitol is implicated in the development of diabetic complications. Members of this enzyme family are also highly expressed in some liver cancers (Cao, D. et al. (1998) J. Biol. Chem. 273: 11429-35).

Alcohol Dehydrogenases

Alcohol dehydrogenases (ADHs) oxidize simple alcohols to the corresponding aldehydes. ADH is a cytosolic enzyme, prefers the cofactor NAD⁺, and also binds zinc ion. Liver contains the highest levels of ADH, with lower levels in kidney, lung, and the gastric mucosa.

Known ADH isoforms are dimeric proteins composed of 40 kDa subunits. There are five known gene loci which encode these subunits (a, b, g, p, c), and some of the loci have characterized allelic variants (b², b³, g¹, g²). The subunits can form homodimers and heterodimers; the subunit composition determines the specific properties of the active enzyme. The holoenzymes have therefore been categorized as Class I (subunit compositions aa, ab, ag, bg, gg), Class II (pp), and Class III (cc). Class I ADH isozymes oxidize ethanol and other small aliphatic alcohols, and are inhibited by pyrazole. Class II isozymes prefer longer chain aliphatic and aromatic alcohols, are unable to oxidize methanol, and are not inhibited by

pyrazole. Class III isozymes prefer even longer chain aliphatic alcohols (five carbons and longer) and aromatic alcohols, and are not inhibited by pyrazole.

The short-chain alcohol dehydrogenases include a number of related enzymes with a variety of substrate specificities. Included in this group are the mammalian enzymes D-beta-hydroxybutyrate dehydrogenase, (R)-3-hydroxybutyrate dehydrogenase, 15-hydroxyprostaglandin dehydrogenase, NADPH-dependent carbonyl reductase, corticosteroid 11-beta-dehydrogenase, and estradiol 17-beta-dehydrogenase, as well as the bacterial enzymes acetoacetyl-CoA reductase, glucose 1-dehydrogenase, 3-beta-hydroxysteroid dehydrogenase, 20-beta-hydroxysteroid dehydrogenase, ribitol dehydrogenase, 3-oxoacyl reductase, 2, 3-dihydro-2, 3-dihydroxybenzoate dehydrogenase, sorbitol-6-phosphate 2-dehydrogenase, 7-alpha-hydroxysteroid dehydrogenase, cis-1, 2-dihydroxy-3, 4-cyclohexadiene-1-carboxylate dehydrogenase, cis-toluene dihydrodiol dehydrogenase, cis-benzene glycol dehydrogenase, biphenyl-2, 3-dihydro-2, 3-diol dehydrogenase, N-acylmannosamine 1-dehydrogenase, and 2-deoxy-D-gluconate 3-dehydrogenase (Krozowski, Z. (1994) J. Steroid Biochem. Mol. Biol. 51: 125-130; Krozowski, Z. (1992) Mol. Cell Endocrinol. 84: C25-31; and Marks, A. R. et al. (1992) J. Biol. Chem. 267: 15459-15463).

UDP Glucuronyltransferase

Members of the UDP glucuronyltransferase family (UGTs) catalyze the transfer of a glucuronic acid group from the cofactor uridine diphosphate-glucuronic acid

(UDP-glucuronic acid) to a substrate. The transfer is generally to a nucleophilic heteroatom (O, N, or S). Substrates include xenobiotics which have been functionalized by Phase I reactions, as well as endogenous compounds such as bilirubin, steroid hormones, and thyroid hormones. Products of glucuronidation are excreted in urine if the molecular weight of the substrate is less than about 250 g/mol, whereas larger glucuronidated substrates are excreted in bile.

UGTs are located in the microsomes of liver, kidney, intestine, skin, brain, spleen, and nasal mucosa, where they are on the same side of the endoplasmic reticulum membrane as cytochrome P450 enzymes and flavin-containing monooxygenases, and therefore are ideally located to access products of Phase I drug metabolism. UGTs have a C-terminal membrane-spanning domain which anchors them in the endoplasmic reticulum membrane and a conserved signature domain of about 50 amino acid residues in their C terminal section.

UGTs involved in drug metabolism are encoded by two gene families, UGT1 and UGT2. Members of the UGT1 family result from alternative splicing of a single gene locus, which has a variable substrate binding domain and constant region involved in cofactor binding and membrane insertion. Members of the UGT2 family are encoded by separate gene loci, and are divided into two families, UGT2A and UGT2B. The 2A subfamily is expressed in olfactory epithelium, and the 2B subfamily is expressed in liver microsomes. Mutations in UGT genes are associated with hyperbilirubinemia; Crigler-Najjar syndrome,

characterized by intense hyperbilirubinemia from birth; and a milder form of hyperbilirubinemia termed Gilbert's disease.

5 **Sulfotransferase**

Sulfate conjugation occurs on many of the same substrates which undergo O-glucuronidation to produce a highly water-soluble sulfuric acid ester. Sulfotransferases (ST) catalyze this reaction by transferring SO_3 -from the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the substrate. ST substrates are predominantly phenols and aliphatic alcohols, but also include aromatic amines and aliphatic amines, which are conjugated to produce the corresponding sulfamates. The products of these reactions are excreted mainly in urine.

STs are found in a wide range of tissues, including liver, kidney, intestinal tract, lung, platelets, and brain. The enzymes are generally cytosolic, and multiple forms are often co-expressed. For example, there are more than a dozen forms of ST in rat liver cytosol. These biochemically characterized STs fall into five classes based on their substrate preference: arylsulfotransferase, alcohol sulfotransferase, estrogen sulfotransferase, tyrosine ester sulfotransferase, and bile salt sulfotransferase.

ST enzyme activity varies greatly with sex and age in rats. The combined effects of developmental cues and sex-related hormones are thought to lead to these differences in ST expression profiles, as well as the profiles of other DMEs such as cytochromes P450. Notably,

the high expression of STs in cats partially compensates for their low level of UDP glucuronyltransferase activity.

Several forms of ST have been purified from human liver cytosol and cloned. There are two phenol
5 sulfotransferases with different thermal stabilities and substrate preferences. The thermostable enzyme catalyzes the sulfation of phenols such as para-nitrophenol, minoxidil, and acetaminophen; the thermolabile enzyme prefers monoamine substrates such as dopamine,
10 epinephrine, and levadopa. Other cloned STs include an estrogen sulfotransferase and an N-acetylglucosamine-6-O-sulfotransferase. This last enzyme is illustrative of the other major role of STs in cellular biochemistry, the modification of carbohydrate structures that may be
15 important in cellular differentiation and maturation of proteoglycans. Indeed, an inherited defect in a sulfotransferase has been implicated in macular corneal dystrophy, a disorder characterized by a failure to synthesize mature keratan sulfate proteoglycans (Nakazawa,
20 K. et al. (1984) J. Biol. Chem. 259: 13751-7).

Galactosyltransferases

Galactosyltransferases are a subset of glycosyltransferases that transfer galactose (Gal) to the
25 terminal N-acetylglucosamine (GlcNAc) oligosaccharide chains that are part of glycoproteins or glycolipids that are free in solution (Kolbinger, F. et al. (1998) J. Biol. Chem. 273: 433-440; Amado, M. et al. (1999) Biochim. Biophys. Acta 1473: 35-53). Galactosyltransferases have
30 been detected on the cell surface and as soluble

extracellular proteins, in addition to being present in the Golgi. β 1,3-galactosyltransferases form Type I carbohydrate chains with Gal (β 1-3) GlcNAc linkages. Known human and mouse β 1,3-galactosyltransferases appear to have

5 a short cytosolic domain, a single transmembrane domain, and a catalytic domain with eight conserved regions. (Kolbinger, F. supra and Hennet, T. et al. (1998) J. Biol. Chem. 273: 58-65). In mouse UDP-galactose : β -N-acetylglucosamine β 1,3-galactosyltransferase-I region 1 is

10 located at amino acid residues 78-83, region 2 is located at amino acid residues 93-102, region 3 is located at amino acid residues 116-119, region 4 is located at amino acid residues 147-158, region 5 is located at amino acid residues 172-183, region 6 is located at amino acid

15 residues 203-206, region 7 is located at amino acid residues 236-246, and region 8 is located at amino acid residues 264-275. A variant of a sequence found within mouse UDP-galactose: β -N-acetylglucosamine β 1,3-galactosyltransferase-I region 8 is also found in

20 bacterial galactosyltransferases, suggesting that this sequence defines a galactosyltransferase sequence motif (Hennet, T. supra). Recent work suggests that brainiac protein is a β 1,3-galactosyltransferase. (Yuan, Y. et al. (1997) Cell 88: 9-11; and Hennet, T. supra).

25 UDP-Gal:GlcNAc-1, 4-galactosyltransferase (-1, 4-GalT) (Sato, T. et al., (1997) EMBO J. 16: 1850-1857) catalyzes the formation of Type II carbohydrate chains with Gal (β 1-4) GlcNAc linkages. As is the case with the β 1,3-galactosyltransferase, a soluble form of the enzyme is

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formed by cleavage of the membrane-bound form. Amino acids conserved among β 1,4-galactosyltransferases include two cysteines linked through a disulfide-bonded and a putative UDPgalactose-binding site in the catalytic domain (Yadav, S. and Brew, K. (1990) J. Biol. Chem. 265: 14163-14169; Yadav, S. P. and Brew, K. (1991) J. Biol. Chem. 266: 698-703; and Shaper, N. L. et al. (1997) J. Biol. Chem. 272: 31389-31399). β 1,4-galactosyltransferases have several specialized roles in addition to synthesizing carbohydrate chains on glycoproteins or glycolipids. In mammals a β 1,4-galactosyltransferase, as part of a heterodimer with cc-lactalbumin, functions in lactating mammary gland lactose production. A β 1,4-galactosyltransferase on the surface of sperm functions as a receptor that specifically recognizes the egg. Cell surface β 1,4-galactosyltransferases also function in cell adhesion, cell/basal lamina interaction, and normal and metastatic cell migration (Shur, B. (1993) Curr. Opin. Cell Biol. 5: 854-863; and Shaper, J. (1995) Adv. Exp. Med. Biol. 376: 95-104).

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Glutathione S-Transferase

The basic reaction catalyzed by glutathione S-transferases (GST) is the conjugation of an electrophile with reduced glutathione (GSH). GSTs are homodimeric or heterodimeric proteins localized mainly in the cytosol, but some level of activity is present in microsomes as well. The major isozymes share common structural and catalytic properties; in humans they have been classified into four major classes, Alpha, Mu, Pi, and Theta. The two

25

largest classes, Alpha and Mu, are identified by their respective protein isoelectric points; pI ~ 7.5-9.0 (Alpha), and pI ~ 6.6 (Mu). Each GST possesses a common binding site for GSH and a variable hydrophobic binding site. The hydrophobic binding site in each isozyme is specific for particular electrophilic substrates. Specific amino acid residues within GSTs have been identified as important for these binding sites and for catalytic activity. Residues Q67, T68, D101, E104, and R131 are important for the binding of GSH (Lee, H-C et al. (1995) J. Biol. Chem. 270: 99-109). Residues R13, R20, and R69 are important for the catalytic activity of GST (Stenberg G et al. (1991) Biochem. J. 274: 549-55).

In most cases, GSTs perform the beneficial function of deactivation and detoxification of potentially mutagenic and carcinogenic chemicals. However, in some cases their action is detrimental and results in activation of chemicals with consequent mutagenic and carcinogenic effects. Some forms of rat and human GSTs are reliable preneoplastic markers that aid in the detection of carcinogenesis. Expression of human GSTs in bacterial strains, such as *Salmonella typhimurium* used in the well-known Ames test for mutagenicity, has helped to establish the role of these enzymes in mutagenesis. Dihalomethanes, which produce liver tumors in mice, are believed to be activated by GST. This view is supported by the finding that dihalomethanes are more mutagenic in bacterial cells expressing human GST than in untransfected cells (Thier, R. et al. (1993) Proc. Natl. Acad. Sci. USA 90: 8567-80). The mutagenicity of ethylene dibromide and ethylene

dichloride is increased in bacterial cells expressing the human Alpha GST, Al-1, while the mutagenicity of allatoxin B1 is substantially reduced by enhancing the expression of GST (Simula, T. P. et al. (1993) Carcinogenesis 14: 1371-6). Thus, control of GST activity may be useful in the control of mutagenesis and carcinogenesis.

GST has been implicated in the acquired resistance of many cancers to drug treatment, the phenomenon known as multi-drug resistance (MDR). MDR occurs when a cancer individual is treated with a cytotoxic drug such as cyclophosphamide and subsequently becomes resistant to this drug and to a variety of other cytotoxic agents as well. Increased GST levels are associated with some of these drug resistant cancers, and it is believed that this increase occurs in response to the drug agent which is then deactivated by the GST catalyzed GSH conjugation reaction. The increased GST levels then protect the cancer cells from other cytotoxic agents which bind to GST. Increased levels of Al-1 in tumors has been linked to drug resistance induced by cyclophosphamide treatment (Dirven H. A. et al. (1994) Cancer Res. 54: 6215-20). Thus control of GST activity in cancerous tissues may be useful in treating MDR in cancer individuals.

Gamma-Glutamyl Transpeptidase

Gamma-glutamyl transpeptidases are ubiquitously expressed enzymes that initiate extracellular glutathione (GSH) breakdown by cleaving gamma-glutamyl amide bonds. The breakdown of GSH provides cells with a regional cysteine pool for biosynthetic pathways. Gamma-glutamyl

transpeptidases also contribute to cellular antioxidant defenses and expression is induced by oxidative stress. The cell surface-localized glycoproteins are expressed at high levels in cancer cells. Studies have suggested that

5 the high level of gamma-glutamyl transpeptidases activity present on the surface of cancer cells could be exploited to activate precursor drugs, resulting in high local concentrations of anticancer therapeutic agents (Hanigan, M. H. (1998) Chem. Biol. Interact. 111-112: 333-42 ;

10 Taniguchi, N. and Ikeda, Y. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72: 239-78 ; Chikhi, N. et al. (1999) Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 122: 367-80).

15 **Acyltransferase**

N-acyltransferase enzymes catalyze the transfer of an amino acid conjugate to an activated carboxylic group. Endogenous compounds and xenobiotics are activated by acyl-CoA synthetases in the cytosol, microsomes, and

20 mitochondria. The acyl-CoA intermediates are then conjugated with an amino acid (typically glycine, glutamin, or taurine, but also ornithine, arginine, histidine, serine, aspartic acid, and several dipeptides) by N-acyltransferases in the cytosol or mitochondria to

25 form a metabolite with an amide bond. This reaction is complementary to O-glucuronidation, but amino acid conjugation does not produce the reactive and toxic metabolites which often result from glucuronidation.

One well-characterized enzyme of this class is the

30 bile acid-CoA : amino acid N-acyltransferase (BAT)

responsible for generating the bile acid conjugates which serve as detergents in the gastrointestinal tract (Falany, C. N. et al. (1994) J. Biol. Chem. 269: 19375-9 ; Johnson, M. R. et al. (1991) J. Biol. Chem. 266: 10227-33). BAT is
5 also useful as a predictive indicator for prognosis of hepatocellular carcinoma individuals after partial hepatectomy (Furutani, M. et al. (1996) Hepatology 24: 1441-5).

10 **Acetyltransferases**

Acetyltransferases have been extensively studied for their role in histone acetylation. Histone acetylation results in the relaxing of the chromatin structure in eukaryotic cells, allowing transcription factors to gain
15 access to promoter elements of the DNA templates in the affected region of the genome (or the genome in general). In contrast, histone deacetylation results in a reduction in transcription by closing the chromatin structure and limiting access of transcription factors. To this end, a
20 common means of stimulating cell transcription is the use of chemical agents that inhibit the deacetylation of histones (e. g., sodium butyrate), resulting in a global (albeit artifactual) increase in gene expression. The modulation of gene expression by acetylation also results
25 from the acetylation of other proteins, including but not limited to, p53, GATA-1, MyoD, ACTR, TFIIE, TFIIIF and the high mobility group proteins (HMG). In the case of p53, acetylation results in increased DNA binding, leading to the stimulation of transcription of genes regulated by
30 p53. The prototypic histone acetylase (HAT) is Gcn5 from

Saccharomyces cerevisiae. Gcn5 is a member of a family of acetylases that includes Tetrahymena p55, human Gcn5, and human p300/CBP. Histone acetylation is reviewed in (Cheung, W. L. et al. (2000) Current Opinion in Cell Biology 12 : 326-333 and Berger, S. L (1999) Current Opinion in Cell Biology 11 : 336-341). Some acetyltransferase enzymes possess the alpha/beta hydrolase fold common to several other major classes of enzymes, including but not limited to, acetylcholinesterases and carboxylesterases.

N-acetyltransferase

Aromatic amines and hydrazine-containing compounds are subject to N-acetylation by the N-acetyltransferase enzymes of liver and other tissues. Some xenobiotics can be O-acetylated to some extent by the same enzymes. N-acetyltransferases are cytosolic enzymes which utilize the cofactor acetyl-coenzyme A (acetyl-CoA) to transfer the acetyl group in a two step process. In the first step, the acetyl group is transferred from acetyl-CoA to an active site cysteine residue; in the second step, the acetyl group is transferred to the substrate amino group and the enzyme is regenerated.

In contrast to most other DME classes, there are a limited number of known N-acetyltransferases. In humans, there are two highly similar enzymes, NAT1 and NAT2; mice appear to have a third form of the enzyme, NAT3. The human forms of N-acetyltransferase have independent regulation (NAT1 is widely-expressed, whereas NAT2 is in liver and gut only) and overlapping substrate preferences. Both

enzymes appear to accept most substrates to some extent, but NAT1 does prefer some substrates (para-aminobenzoic acid, para-aminosalicylic acid, sulfamethoxazole, and sulfanilamide), while NAT2 prefers others (isoniazid, 5 hydralazine, procainamide, dapsone, aminogluthethimide, and sulfamethazine).

Clinical observations of individuals taking the antituberculosis drug isoniazid in the 1950s led to the description of fast and slow acetylators of the compound. 10 These phenotypes were shown subsequently to be due to mutations in the NAT2 gene which affected enzyme activity or stability. The slow isoniazid acetylator phenotype is very prevalent in Middle Eastern populations (approx. 70%), and is less prevalent in Caucasian (approx. 50%) and 15 Asian (<25%) populations. More recently, functional polymorphism in NAT1 has been detected, with approximately 8% of the population tested showing a slow acetylator phenotype (Butcher, N. J. et al. (1998) Pharmacogenetics 8: 67-72). Since NAT1 can activate some known aromatic 20 amine carcinogens, polymorphism in the widely-expressed NAT1 enzyme may be important in determining cancer risk.

Aminotransferases

Aminotransferases comprise a family of pyridoxal 25 5'-phosphate (PLP)-dependent enzymes that catalyze transformations of amino acids. Aspartate aminotransferase (AspAT) is the most extensively studied PLP-containing enzyme. It catalyzes the reversible transamination of dicarboxylic L-amino acids, aspartate and glutamate, and 30 the corresponding 2-oxo acids, oxalacetate and 2-

oxoglutarate. Other members of the family included pyruvate aminotransferase, branched-chain amino acid aminotransferase, tyrosine aminotransferase, aromatic aminotransferase, alanine : glyoxylate aminotransferase (AGT), and kynurenine aminotransferase (Vacca, R. A. et al. (1997) J. Biol. Chem. 272: 21932-21937).

Primary hyperoxaluria type-1 is an autosomal recessive disorder resulting in a deficiency in the liver-specific peroxisomal enzyme, alanine : glyoxylate aminotransferase-1. The phenotype of the disorder is a deficiency in glyoxylate metabolism. In the absence of AGT, glyoxylate is oxidized to oxalate rather than being transaminated to glycine. The result is the deposition of insoluble calcium oxalate in the kidneys and urinary tract, ultimately causing renal failure (Lumb, M. J. et al. (1999) J. Biol. Chem. 274: 20587-20596).

Kynurenine aminotransferase catalyzes the irreversible transamination of the L-tryptophan metabolite L-kynurenine to form kynurenic acid. The enzyme may also catalyze the reversible transamination reaction between L-2-aminoadipate and 2-oxoglutarate to produce 2-oxoadipate and L-glutamate. Kynurenic acid is a putative modulator of glutamatergic neurotransmission, thus a deficiency in kynurenine aminotransferase may be associated with pleiotropic effects (Buchli, R. et al. (1995) J. Biol. Chem. 270: 29330-29335).

Catechol-O-Methyltransferase

Catechol-O-methyltransferase (COMT) catalyzes the transfer of the methyl group of S-adenosylmethionine

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(AdoMet ; SAM) donor to one of the hydroxyl groups of the catechol substrate (e.g., L-dopa, dopamine, or DBA). Methylation of the 3'-hydroxyl group is favored over methylation of the 4'-hydroxyl group and the membrane bound isoform of COMT is more regiospecific than the soluble form. Translation of the soluble form of the enzyme results from utilization of an internal start codon in a full-length mRNA (1.5 kb) or from the translation of a shorter mRNA (1.3 kb), transcribed from an internal promoter. The proposed S_N2 -like methylation reaction requires Mg^{2+} and is inhibited by Ca^{2+} . The binding of the donor and substrate to COMT occurs sequentially. AdoMet first binds COMT in a Mg^{2+} -independent manner, followed by the binding of Mg^{2+} and the binding of the catechol substrate.

The amount of COMT in tissues is relatively high compared to the amount of activity normally required, thus inhibition is problematic. Nonetheless, inhibitors have been developed for *in vitro* use (e.g., galates, tropolone, U-0521, and 3',4'-dihydroxy-2-methyl-propiophetropolone) and for clinical use (e.g., nitrocatechol-based compounds and tolcapone). Administration of these inhibitors results in the increased half-life of L-dopa and the consequent formation of dopamine. Inhibition of COMT is also likely to increase the half-life of various other catechol-structure compounds, including but not limited to epinephrine/norepinephrine, isoprenaline, rimiterol, dobutamine, fenoldopam, apomorphine, and α -methyldopa. A deficiency in norepinephrine has been linked to clinical depression, hence the use of COMT inhibitors could be

useful in the treatment of depression. COMT inhibitors are generally well tolerated with minimal side effects and are ultimately metabolized in the liver with only minor accumulation of metabolites in the body (Männistö, P. T. and Kaakkola, S. (1999) *Pharmacological Reviews* 51: 593-628).

Copper-Zinc Superoxide Dismutases

Copper-zinc superoxide dismutases are compact homodimeric metalloenzymes involved in cellular defenses against oxidative damage. The enzymes contain one atom of zinc and one atom of copper per subunit and catalyze the dismutation of superoxide anions into O_2 and H_2O_2 . The rate of dismutation is diffusion-limited and consequently enhanced by the presence of favorable electrostatic interactions between the substrate and enzyme active site. Examples of this class of enzyme have been identified in the cytoplasm of all the eukaryotic cells as well as in the periplasm of several bacterial species. Copper-zinc superoxide dismutases are robust enzymes that are highly resistant to proteolytic digestion and denaturing by urea and SDS. In addition to the compact structure of the enzymes, the presence of the metal ions and intrasubunit disulfide bonds is believed to be responsible for enzyme stability. The enzymes undergo reversible denaturation at temperatures as high as $70^\circ C$ (Battistoni, A. et al. (1998) *J. Biol. Chem.* 273:655-5661).

Overexpression of superoxide dismutase has been implicated in enhancing freezing tolerance of transgenic Alfalfa as well as providing resistance to environmental

toxins such as the diphenyl ether herbicide, acifluorfen (McKersie, B. D. et al. (1993) Plant Physiol. 103: 1155-1163). In addition, yeast cells become more resistant to freeze-thaw damage following exposure to hydrogen peroxide which causes the yeast cells to adapt to further peroxide stress by upregulating expression of superoxide dismutases. In this study, mutations to yeast superoxide dismutase genes had a more detrimental effect on freeze-thaw resistance than mutations which affected the regulation of glutathione metabolism, long suspected of being important in determining an organism's survival through the process of cryopreservation (Jong-In Park, J-I. et al. (1998) J. Biol. Chem. 273: 22921-22928).

Expression of superoxide dismutase is also associated with *Mycobacterium tuberculosis*, the organism that causes tuberculosis. Superoxide dismutase is one of the ten major proteins secreted by *M. tuberculosis* and its expression is upregulated approximately 5-fold in response to oxidative stress. *M. tuberculosis* expresses almost two orders of magnitude more superoxide dismutase than the nonpathogenic mycobacterium *M. smegmatis*, and secretes a much higher proportion of the expressed enzyme. The result is the secretion of 350-fold more enzyme by *M. tuberculosis* than *M. smegmatis*, providing substantial resistance to oxidative stress (Harth, G. and Horwitz, M. A. (1999) J. Biol. Chem. 274: 4281-4292).

The reduced expression of copper-zinc superoxide dismutases, as well as other enzymes with anti-oxidant capabilities, has been implicated in the early stages of cancer. The expression of copper-zinc superoxide

dismutases has been shown to be lower in prostatic intraepithelial neoplasia and prostate carcinomas, compared to normal prostate tissue (Bostwick, D. G. (2000) Cancer 89: 123-134).

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Phosphodiesterases

Phosphodiesterases make up a class of enzymes which catalyze the hydrolysis of one of the two ester bonds in a phosphodiester compound. Phosphodiesterases are therefore crucial to a variety of cellular processes. Phosphodiesterases include DNA and RNA endonucleases and exonucleases, which are essential for cell growth and replication, and topoisomerases, which break and rejoin nucleic acid strands during topological rearrangement of DNA. A Tyr-DNA phosphodiesterase functions in DNA repair by hydrolyzing dead-end covalent intermediates formed between topoisomerase I and DNA (Pouliot, J. J. et al. (1999) Science 286: 552-555; Yang, S.-W. (1996) Proc. Natl. Acad. Sci. USA 93: 11534-11539).

Acid sphingomyelinase is a phosphodiesterase which hydrolyzes the membrane phospholipid sphingomyelin to produce ceramide and phosphorylcholine. Phosphorylcholine is used in the synthesis of phosphatidylcholine, which is involved in numerous intracellular signaling pathways, while ceramide is an essential precursor for the generation of gangliosides, membrane lipids found in high concentration in neural tissue. Defective acid sphingomyelinase leads to a build-up of sphingomyelin molecules in lysosomes, resulting in Niemann-Pick disease

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(Schuchman, E. H. and S. R. Miranda (1997) Genet. Test. 1: 13-19).

Glycerophosphoryl diester phosphodiesterase (also known as glycerophosphodiester phosphodiesterase) is a phosphodiesterase which hydrolyzes deacetylated phospholipid glycerophosphodiester to produce sn-glycerol-3-phosphate and an alcohol. Glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoglycerol, and glycerophosphoinositol are examples of substrates for glycerophosphoryl diester phosphodiesterases. A glycerophosphoryl diester phosphodiesterase from *E. coli* has broad specificity for glycerophosphodiester substrates (Larson, T. J. et al. (1983) J. Biol. Chem. 248: 5428-5432).

Cyclic nucleotide phosphodiesterases (PDEs) are crucial enzymes in the regulation of the cyclic nucleotides cAMP and cGMP. cAMP and cGMP function as intracellular second messengers to transduce a variety of extracellular signals including hormones, light, and neurotransmitters. PDEs degrade cyclic nucleotides to their corresponding monophosphates, thereby regulating the intracellular concentrations of cyclic nucleotides and their effects on signal transduction. Due to their roles as regulators of signal transduction, PDEs have been extensively studied as chemotherapeutic targets (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-81; Torphy, J. T. (1998) Am. J. Resp. Crit. CareMed. 157: 351-370).

Families of mammalian PDEs have been classified based on their substrate specificity and affinity,

sensitivity to cofactors, and sensitivity to inhibitory agents (Beavo, J. A. (1995) *Physiol. Rev.* 75: 725-748; Conti, M. et al. (1995) *Endocrine Rev.* 16: 370-389). Several of these families contain distinct genes, many of which are expressed in different tissues as splice variants. Within PDE families, there are multiple isozymes and multiple splice variants of these isozymes (Conti, M. and S. L. C. Jin (1999) *Prog. Nucleic Acid Res. Mol. Biol.* 63: 1-38). The existence of multiple PDE families, isozymes, and splice variants is an indication of the variety and complexity of the regulatory pathways involving cyclic nucleotides (Houslay, M. D. and G. Milligan (1997) *Trends Biochem. Sci.* 22: 217-224).

Type 1 PDEs (PDE1s) are Ca^{2+} /calmodulin-dependent and appear to be encoded by at least three different genes, each having at least two different splice variants (Kakkar, R. et al. (1999) *Cell Mol. Life Sci.* 55: 1164-1186). PDE1s have been found in the lung, heart, and brain. Some PDE1 isozymes are regulated *in vitro* by phosphorylation/dephosphorylation. Phosphorylation of these PDE1 isozymes decreases the affinity of the enzyme for calmodulin, decreases PDE activity, and increases steady state levels of cAMP (Kakkar, *supra*). PDE1s may provide useful therapeutic targets for disorders of the central nervous system, and the cardiovascular and immune systems due to the involvement of PDE1s in both cyclic nucleotide and calcium signaling (Perry, M. J. and G. A. Higgs (1998) *Curr. Opin. Chem. Biol.* 2: 472-481).

PDE2s are cGMP-stimulated PDEs that have been found in the cerebellum, neocortex, heart, kidney, lung,

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pulmonary artery, and skeletal muscle (Sadhu, K. et al. (1999) J. Histochem. Cytochem. 47: 895-906). PDE2s are thought to mediate the effects of cAMP on catecholamine secretion, participate in the regulation of aldosterone
5 (Beavo, *supra*), and play a role in olfactory signal transduction (Juilfs, D. M. et al. (1997) Proc. Natl. Acad. Sci. USA 94: 3388-3395).

PDE3s have high affinity for both cGMP and cAMP, and so these cyclic nucleotides act as competitive
10 substrates for PDE3s. PDE3s play roles in stimulating myocardial contractility, inhibiting platelet aggregation, relaxing vascular and airway smooth muscle, inhibiting proliferation of T-lymphocytes and cultured vascular smooth muscle cells, and regulating catecholamine-induced
15 release of free fatty acids from adipose tissue. The PDE3 family of phosphodiesterases are sensitive to specific inhibitors such as cilostamide, enoximone, and lixazinone. Isozymes of PDE3 can be regulated by cAMP-dependent protein kinase, or by insulin-dependent kinases (Degerman,
20 E. et al. (1997) J. Biol. Chem. 272: 6823-6826).

PDE4s are specific for cAMP, are localized to airway smooth muscle, the vascular endothelium, and all inflammatory cells; and can be activated by cAMP-dependent phosphorylation. Since elevation of cAMP levels can lead
25 to suppression of inflammatory cell activation and to relaxation of bronchial smooth muscle, PDE4s have been studied extensively as possible targets for novel anti-inflammatory agents, with special emphasis placed on the discovery of asthma treatments. PDE4 inhibitors are
30 currently undergoing clinical trials as treatments for

asthma, chronic obstructive pulmonary disease, and atopic eczema. All four known isozymes of PDE4 are susceptible to the inhibitor rolipram, a compound which has been shown to improve behavioral memory in mice (Barad, M. et al. (1998) Proc. Natl. Acad. Sci. USA 95: 15020-15025). PDE4 inhibitors have also been studied as possible therapeutic agents against acute lung injury, endotoxemia, rheumatoid arthritis, multiple sclerosis, and various neurological and gastrointestinal indications (Doherty, A. M. (1999) Curr. Opin. Chem. Biol. 3: 466-473).

PDE5 is highly selective for cGMP as a substrate (Turko, I. V. et al. (1998) Biochemistry 37: 4200-4205), and has two allosteric cGMP-specific binding sites (McAllister-Lucas, L. M. et al. (1995) J. Biol. Chem. 270: 30671-30679). Binding of cGMP to these allosteric binding sites seems to be important for phosphorylation of PDE5 by cGMP-dependent protein kinase rather than for direct regulation of catalytic activity. High levels of PDE5 are found in vascular smooth muscle, platelets, lung, and kidney. The inhibitor zaprinast is effective against PDE5 and PDE1s. Modification of zaprinast to provide specificity against PDE5 has resulted in sildenafil (VIAGRA; Pfizer, Inc., New York NY), a treatment for male erectile dysfunction (Terrett, N. et al. (1996) Bioorg. Med. Chem. Lett. 6: 1819-1824). Inhibitors of PDE5 are currently being studied as agents for cardiovascular therapy (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481).

PDE6s, the photoreceptor cyclic nucleotide phosphodiesterases, are crucial components of the

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phototransduction cascade. In association with the G-protein transducin, PDE6s hydrolyze cGMP to regulate cGMP-gated cation channels in photoreceptor membranes. In addition to the cGMP-binding active site, PDE6s also have
5 two high-affinity cGMP-binding sites which are thought to play a regulatory role in PDE6 function (Artemyev, N. O. et al. (1998) Methods 14: 93-104). Defects in PDE6s have been associated with retinal disease. Retinal degeneration in the rd mouse (Yan, W. et al. (1998) Invest. Ophthalmol.
10 Vis. Sci. 39: 2529-2536), autosomal recessive retinitis pigmentosa in humans (Danciger, M. et al. (1995) Genomics 30: 1-7), and rod/cone dysplasia 1 in Irish Setter dogs (Suber, M. L. et al. (1993) Proc. Natl. Acad. Sci. USA 90: 3968- 972) have been attributed to mutations in the PDE6B
15 gene.

The PDE7 family of PDEs consists of only one known member having multiple splice variants (Bloom, T. J. and J. A. Beavo (1996) Proc. Natl. Acad. Sci. USA 93: 14188-14192). PDE7s are cAMP specific, but little else is known
20 about their physiological function. Although mRNAs encoding PDE7s are found in skeletal muscle, heart, brain, lung, kidney, and pancreas, expression of PDE7 proteins is restricted to specific tissue types (Han, P. et al. (1997) J. Biol. Chem. 272: 16152-16157 ; Perry, M. J. and G. A.
25 Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481). PDE7s are very closely related to the PDE4 family; however, PDE7s are not inhibited by rolipram, a specific inhibitor of PDE4s (Beavo, *supra*).

PDE8s are cAMP specific, and are closely related
30 to the PDE4 family. PDE8s are expressed in thyroid gland,

testis, eye, liver, skeletal muscle, heart, kidney, ovary, and brain. The cAMP hydrolyzing activity of PDE8s is not inhibited by the PDE inhibitors rolipram, vinpocetine, milrinone, IBMX (3-isobutyl-1-methylxanthine), or zaprinast, but PDE8s are inhibited by dipyridamole (Fisher, D. A. et al. (1998) Biochem. Biophys. Res. Commun. 246: 570-577 ; Hayashi, M. et al. (1998) Biochem. Biophys. Res. Commun. 250: 751-756 ; Soderling, S. H. et al. 1998) Proc. Natl. Acad. Sci. USA 95: 8991-8996).

PDE9s are cGMP specific and most closely resemble the PDE8 family of PDEs. PDE9s are expressed in kidney, liver, lung, brain, spleen, and small intestine. PDE9s are not inhibited by sildenafil (VIAGRA; Pfizer, Inc., New York NY), rolipram, vinpocetine, dipyridamole, or IBMX (3-isobutyl-1-methylxanthine), but they are sensitive to the PDE5 inhibitor zaprinast (Fisher, D. A. et al. (1998) J. Biol. Chem. 273: 15559-15564 ; Soderling, S. H. et al. (1998) J. Biol. Chem. 273: 15553-15558).

PDE10s are dual-substrate PDEs, hydrolyzing both cAMP and cGMP. PDE10s are expressed in brain, thyroid, and testis. (Soderling, S. H. et al. (1999) Proc. Natl. Acad. Sci. USA 96: 7071-7076; Fujishige, K. et al. (1999) J. Biol. Chem. 274: 18438-18445; Loughney, K. et al. (1999) Gene 234: 109117).

PDEs are composed of a catalytic domain of about 270-300 amino acids, an N-terminal regulatory domain responsible for binding cofactors, and, in some cases, a hydrophilic C-terminal domain of unknown function (Conti, M. and S.-L. C. Jin (1999) Prog. Nucleic Acid Res. Mol. Biol. 63: 1-38). A conserved, putative zinc-binding motif,

HDXXHXGXXN, has been identified in the catalytic domain of all PDEs. N-terminal regulatory domains include non-catalytic cGMP-binding domains in PDE2s, PDE5s, and PDE6s; calmodulin-binding domains in PDE1s; and domains
5 containing phosphorylation sites in PDE3s and PDE4s. In PDE5, the N-terminal cGMP-binding domain spans about 380 amino acid residues and comprises tandem repeats of the conserved sequence motif N (R/K) XnFX3DE (McAllister-Lucas, L. M. et al. (1993) J. Biol. Chem. 268: 22863-
10 22873). The NKXnD motif has been shown by mutagenesis to be important for cGMP binding (Turko, I. V. et al. (1996) J. Biol. Chem. 271: 22240-22244). PDE families display approximately 30% amino acid identity within the catalytic domain; however, isozymes within the same family typically
15 display about 85-95% identity in this region (e.g. PDE4A vs PDE4B). Furthermore, within a family there is extensive similarity (>60%) outside the catalytic domain; while across families, there is little or no sequence similarity outside this domain.

20 Many of the constituent functions of immune and inflammatory responses are inhibited by agents that increase intracellular levels of cAMP (Verghese, M. W. et al. (1995) Mol. Pharmacol. 47: 1164-1171). A variety of diseases have been attributed to increased PDE activity
25 and associated with decreased levels of cyclic nucleotides. For example, a form of diabetes insipidus in mice has been associated with increased PDE4 activity, an increase in low- K_m cAMP PDE activity has been reported in leukocytes of atopic individuals, and PDE3 has been
30 associated with cardiac disease.

Many inhibitors of PDEs have been identified and have undergone clinical evaluation (Perry, M. J. and G. A. Higgs (1998) *Curr. Opin. Chem. Biol.* 2: 472-481; Torphy, T. J. (1998) *Am. J. Respir. Crit. Care Med.* 157: 351-370).

5 PDE3 inhibitors are being developed as antithrombotic agents, antihypertensive agents, and as cardiotonic agents useful in the treatment of congestive heart failure. Rolipram, a PDE4 inhibitor, has been used in the treatment of depression, and other inhibitors of PDE4 are undergoing

10 evaluation as anti-inflammatory agents. Rolipram has also been shown to inhibit lipopolysaccharide (LPS) induced TNF- α , which has been shown to enhance HIV-1 replication in vitro. Therefore, rolipram may inhibit HIV-1 replication (Angel, J. B. et al. (1995) *AIDS* 9: 1137-

15 1144). Additionally, rolipram, based on its ability to suppress the production of cytokines such as TNF- α and b and interferon γ , has been shown to be effective in the treatment of encephalomyelitis. Rolipram may also be effective in treating tardive dyskinesia and was effective

20 in treating multiple sclerosis in an experimental animal model (Sommer, N. et al. (1995) *Nat. Med.* 1: 244-248; Sasaki, H. et al. (1995) *Eur. J. Pharmacol.* 282: 71-76).

Theophylline is a nonspecific PDE inhibitor used in the treatment of bronchial asthma and other respiratory

25 diseases. Theophylline is believed to act on airway smooth muscle function and in an anti-inflammatory or immunomodulatory capacity in the treatment of respiratory diseases (Banner, K. H. and C. P. Page (1995) *Eur. Respir. J.* 8: 996-1000). Pentoxifylline is another nonspecific PDE

30 inhibitor used in the treatment of intermittent

claudication and diabetes-induced peripheral vascular disease. Pentoxifylline is also known to block TNF- α production and may inhibit HIV-1 replication (Angel et al., *supra*).

5 PDEs have been reported to affect cellular proliferation of a variety of cell types (Conti et al. (1995) Endocrine Rev. 16: 370-389) and have been implicated in various cancers. Growth of prostate carcinoma cell lines DU145 and LNCaP was inhibited by
10 delivery of cAMP derivatives and PDE inhibitors (Bang, Y. J. et al. (1994) Proc. Natl. Acad. Sci. USA 91: 5330-5334). These cells also showed a permanent conversion in phenotype from epithelial to neuronal morphology. It has also been suggested that PDE inhibitors have the potential
15 to regulate mesangial cell proliferation (Matousovic, K. et al. (1995) J. Clin. Invest. 96: 401-410) and lymphocyte proliferation (Joulain, C. et al. (1995) J. Lipid Mediat. Cell Signal. 11: 63-79). A cancer treatment has been described that involves intracellular delivery of PDEs to
20 particular cellular compartments of tumors, resulting in cell death (Deonarain, M. P. and A. A. Epenetos (1994) Br. J. Cancer 70: 786-794).

Phosphotriesterases

25 Phosphotriesterases (PTE, paraoxonases) are enzymes that hydrolyze toxic organophosphorus compounds and have been isolated from a variety of tissues. The enzymes appear to be lacking in birds and insects, but is abundant in mammals, explaining the reduced tolerance of
30 birds and insects to organophosphorus compound (Vilanova,

E. and Sogorb, M. A. (1999) Crit. Rev. Toxicol. 29: 21-57). Phosphotriesterases play a central role in the detoxification of insecticides by mammals. Phosphotriesterase activity varies among individuals and is lower in infants than adults. Knockout mice are markedly more sensitive to the organophosphate-based toxins diazoxon and chlorpyrifos oxon (Furlong, C. E., et al. (2000) Neurotoxicology 21: 91-100). PTEs have attracted interest as enzymes capable of the detoxification of organophosphate-containing chemical waste and warfare reagents (e.g., parathion), in addition to pesticides and insecticides. Some studies have also implicated phosphotriesterase in atherosclerosis and diseases involving lipoprotein metabolism.

15

Thioesterases

Two soluble thioesterases involved in fatty acid biosynthesis have been isolated from mammalian tissues, one which is active only toward long-chain fatty-acyl thioesters and one which is active toward thioester with a wide range of fatty-acyl chain-lengths. These thioesterases catalyze the chain-terminating step in the de novo biosynthesis of fatty acids. Chain termination involves the hydrolysis of the thioester bond which links the fatty acyl chain to the 4'-phosphopantetheine prosthetic group of the acyl carrier protein (ACP) subunit of the fatty acid synthase (Smith, S. (1981a) Methods Enzymol. 71: 181-188; Smith, S. (1981b) Methods Enzymol. 71: 188-200).

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E. coli contains two soluble thioesterases, thioesterase I (TEI) which is active only toward long-chain acyl thioesters, and thioesterase II (TEII) which has a broad chain-length specificity (Naggert, J. et al. (1991) J. Biol. Chem. 266: 11044-11050). *E. coli* TEII does not exhibit sequence similarity with either of the two types of mammalian thioesterases which function as chain-terminating enzymes in *de novo* fatty acid biosynthesis. Unlike the mammalian thioesterases, *E. coli* TEII lacks the characteristic serine active site gly-X-ser-X-gly sequence motif and is not inactivated by the serine modifying agent diisopropyl fluorophosphate. However, modification of histidine 58 by iodoacetamide and diethylpyrocarbonate abolished TEII activity. Overexpression of TEII did not alter fatty acid content in *E. coli*, which suggests that it does not function as a chain-terminating enzyme in fatty acid biosynthesis (Naggert et al., *supra*). For that reason, Naggert et al. (*supra*) proposed that the physiological substrates for *E. coli* TEII may be coenzyme A (CoA)-fatty acid esters instead of ACP-phosphopanthetheine-fatty acid esters.

Carboxylesterases

Mammalian carboxylesterases constitute a multigene family expressed in a variety of tissues and cell types. Isozymes have significant sequence homology and are classified primarily on the basis of amino acid sequence. Acetylcholinesterase, butyrylcholinesterase, and carboxylesterase are grouped into the serine super family of esterases (B-esterases). Other carboxylesterases

included thyroglobulin, thrombin, Factor IX, gliotactin, and plasminogen. Carboxylesterases catalyze the hydrolysis of ester and amide-groups from molecules and are involved in detoxification of drugs, environmental toxins, and carcinogens. Substrates for carboxylesterases include short-and long-chain acyl-glycerols, acylcarnitine, carbonates, dipivefrin hydrochloride, cocaine, salicylates, capsaicin, palmitoyl-coenzyme A, imidapril, haloperidol, pyrrolizidine alkaloids, steroids, p-nitrophenyl acetate, malathion, butanilicaine, and isocarboxazide. The enzymes often demonstrate low substrate specificity. Carboxylesterases are also important for the conversion of prodrugs to their respective free acids, which may be the active form of the drug (e.g., lovastatin, used to lower blood cholesterol) (reviewed in Satoh, T. and Hosokawa, M. (1998) *Annu. Rev. Pharmacol. Toxicol.* 38: 257-288).

Neuroligins are a class of molecules that (i) have N-terminal signal sequences, (ii) resemble cell-surface receptors, (iii) contain carboxylesterase domains, (iv) are highly expressed in the brain, and (v) bind to neurexins in a calcium-dependent manner. Despite the homology to carboxylesterases, neuroligins lack the active site serine residue, implying a role in substrate binding rather than catalysis (Ichtchenko, K. et al. (1996) *J. Biol. Chem.* 271: 2676-2682).

Squalene Epoxidase

Squalene epoxidase (squalene monooxygenase, SE) is a microsomal membrane-bound, FAD-dependent oxidoreductase

that catalyzes the first oxygenation step in the sterol biosynthetic pathway of eukaryotic cells. Cholesterol is an essential structural component of cytoplasmic membranes acquired via the LDL receptor-mediated pathway or the biosynthetic pathway. In the latter case, all 27 carbon atoms in the cholesterol molecule are derived from acetyl-CoA (Stryer, L., *supra*). SE converts squalene to 2, 3 (S)-oxidosqualene, which is then converted to lanosterol and then cholesterol. The steps involved in cholesterol biosynthesis are summarized below (Stryer, L (1988) Biochemistry. W. H Freeman and Co., Inc. New York. pp. 554-560 and Sakakibara, J. et al. (1995) 270: 17-20) :

acetate (from Acetyl-CoA)	3-hydroxy-3-methyl-glutaryl CoA
mevalonate	5-phosphomevalonate
pyrophosphomevalonate	isopentenyl pyrophosphate
dimethylallyl pyrophosphate	geranyl pyrophosphate
farnesyl pyrophosphate	squalene
lanosterol	cholesterol.

While cholesterol is essential for the viability of eukaryotic cells, inordinately high serum cholesterol levels results in the formation of atherosclerotic plaques in the arteries of higher organisms. This deposition of highly insoluble lipid material onto the walls of essential blood vessels (e.g., coronary arteries) results in decreased blood flow and potential necrosis of the tissues deprived of adequate blood flow. HMG-CoA reductase is responsible for the conversion of 3-hydroxyl-3-methylglutaryl CoA (HMG-CoA) to mevalonate, which

represents the first committed step in cholesterol biosynthesis. HMG-CoA is the target of a number of pharmaceutical compounds designed to lower plasma cholesterol levels. However, inhibition of MHG-CoA also
5 results in the reduced synthesis of non-sterol intermediates (e.g., mevalonate) required for other biochemical pathways. SE catalyzes a rate-limiting reaction that occurs later in the sterol synthesis pathway and cholesterol is the only end product of the pathway
10 following the step catalyzed by SE. As a result, SE is the ideal target for the design of anti-hyperlipidemic drugs that do not cause a reduction in other necessary intermediates (Nakamura, Y. et al. (1996) 271: 8053-8056).

15 **Epoxide hydrolases**

Epoxide hydrolases catalyze the addition of water to epoxide-containing compounds, thereby hydrolyzing epoxides to their corresponding 1, 2-diols. They are related to bacterial haloalkane dehalogenases and show
20 sequence similarity to other members of the α/β hydrolase fold family of enzymes (e.g., bromoperoxidase A2 from *Streptomyces aureofaciens*, hydroxymuconic semialdehyde hydrolases from *Pseudomonas putida*, and haloalkane dehalogenase from *Xanthobacter autotrophicus*). Epoxide
25 hydrolases are ubiquitous in nature and have been found in mammals, invertebrates, plants, fungi, and bacteria. This family of enzymes is important for the detoxification of xenobiotic epoxide compounds which are often highly electrophilic and destructive when introduced into an
30 organism. Examples of epoxide hydrolase reactions include

the hydrolysis of cis-9, 10-epoxyoctadec-9 (Z)-enoic acid (leukotoxin) to form its corresponding diol, threo-9, 10-dihydroxyoctadec-12 (Z)-enoic acid (leukotoxin diol), and the hydrolysis of cis-12, 13-epoxyoctadec-9 (Z)-enoic acid (isoleukotoxin) to form its corresponding diol threo-12, 13-dihydroxyoctadec-9 (Z)-enoic acid (isoleukotoxin diol). Leukotoxins alter membrane permeability and ion transport and cause inflammatory responses. In addition, epoxide carcinogens are known to be produced by cytochrome P450 as intermediates in the detoxification of drugs and environmental toxins.

The enzymes possess a catalytic triad composed of Asp (the nucleophile), Asp (the histidine-supporting acid), and His (the water-activating histidine). The reaction mechanism of epoxide hydrolase proceeds via a covalently bound ester intermediate initiated by the nucleophilic attack of one of the Asp residues on the primary carbon atom of the epoxide ring of the target molecule, leading to a covalently bound ester intermediate (Michael Arand, M. et al. (1996) J. Biol. Chem. 271: 4223-4229; Rink, R. et al. (1997) J. Biol. Chem. 272: 14650-14657; Argiriadi, M. A. et al. (2000) J. Biol. Chem. 275: 15265-15270).

Enzymes involved in tyrosine catalysis

The degradation of the amino acid tyrosine to either succinate and pyruvate or fumarate and acetoacetate, requires a large number of enzymes and generates a large number of intermediate compounds. In addition, many xenobiotic compounds may be metabolized

using one or more reactions that are part of the tyrosine catabolic pathway. While the pathway has been studied primarily in bacteria, tyrosine degradation is known to occur in a variety of organisms and is likely to involve
5 many of the same biological reactions.

The enzymes involved in the degradation of tyrosine to succinate and pyruvate (e.g., in *Artlirobacter* species) include 4-hydroxyphenylpyruvate oxidase, 4-hydroxyphenylacetate 3-hydroxylase, 3, 4-
10 dihydroxyphenylacetate 2, 3-dioxygenase, 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase, trans, cis-5-carboxymethyl-2-hydroxymuconate isomerase, homoprotocatechuate isomerase/decarboxylase, cis-2-oxohept-3-ene-1, 7-dioate hydratase, 2, 4-dihydroxyhept-
15 trans-2-ene-1, 7-dioate aldolase, and succinic semialdehyde dehydrogenase.

The enzymes involved in the degradation of tyrosine to fumarate and acetoacetate (e.g., in *Pseudomonas* species) include 4-hydroxyphenylpyruvate
20 dioxygenase, homogentisate 1, 2-dioxygenase, maleylacetoacetate isomerase, and fumarylacetoacetase. 4-hydroxyphenylacetate 1-hydroxylase may also be involved if intermediates from the succinate/pyruvate pathway are accepted.

25 Additional enzymes associated with tyrosine metabolism in different organisms include 4-chlorophenylacetate-3, 4-dioxygenase, aromatic aminotransferase, 5-oxopent-3-ene-1, 2, 5-tricarboxylate decarboxylase, 2-oxo-hept-3-ene-1, 7-dioate hydratase, and
30 5-carboxymethyl-2-hydroxymuconate isomerase (Ellis, L. B.

M. et al. (1999) Nucleic Acids Res. 27: 373-376; Wackett, L. P. and Ellis, L. B. M. (1996) J. Microbiol. Meth. 25: 91-93; and Schmidt, M. (1996) Amer. Soc. Microbiol. News 62: 102).

5 In humans, acquired or inherited genetic defects in enzymes of the tyrosine degradation pathway may result in hereditary tyrosinemia. One form of this disease, hereditary tyrosinemia 1 (HT1) is caused by a deficiency in the enzyme fumarylacetoacetate hydrolase, the last
10 enzyme in the pathway in organisms that metabolize tyrosine to fumarate and acetoacetate. HT1 is characterized by progressive liver damage beginning at infancy, and increased risk for liver cancer (Endo, F. et al. (1997) J. Biol. Chem. 272: 24426-24432).

15 An enzyme of one system can act on several drugs and drug metabolites. The rate of metabolism of a drug differs between individuals and between ethnic groups, owing to the existence of enzymatic polymorphism within each system. Metabolic phenotypes have been generally
20 characterized as poor metabolizers (PM), extensive metabolizers (EM), and ultra-extensive metabolizers (UEM). Knowledge of a metabolic phenotype is clinically useful for the following reasons:

- 25 1) a phenotype may be correlated to an individual's susceptibility to toxic chemicals, diseases and cancers;
- 2) a phenotype may provide a physician with valuable information for quickly determining a safe and therapeutically-effective drug
30 treatment regimen for an individual; and

- 3) individual phenotypes may provide valuable rationales for the design of therapeutic drugs.

To date, the ability to characterize multiple phenotypic determinants for the purpose of identifying individual phenotypes, drug treatment compatibility and susceptibility has been limited by the complexities of multiple metabolic pathways, and the lack of efficient and effective procedures for making these determinations. Currently, the determination of an individual's phenotype for a given metabolic enzyme can be performed either via direct metabolic phenotyping or indirect extrapolation of an individual's genotype to a given phenotype.

Direct phenotyping involves the use a probe substrate known to be metabolized by a given enzyme. The rate of metabolism of the probe substrate is measured and this rate of metabolism is used to determine a metabolic phenotype. Although labor intensive and costly procedures for direct phenotyping have been known for many years these procedures are not readily adaptable for a clinical environment, nor are they practical for measuring multiple phenotypic determinants. For example, enzymatic phenotypes may be determined by measurements of the molar (or chiral) ratio of metabolites of a drug or a probe substrate in a urine sample from a individual by high-pressure liquid chromatography (HPLC), capillary electrophoresis (CE) or stereo-selective capillary gas chromatography. These determination methods are time-consuming, onerous, and employ systems and equipment that are not readily available in a clinical laboratory. Methodologies for the

rapid determination of multiple determinants of a metabolic phenotypic are not available, and as a result, valuable information concerning an individual's phenotype is not considered on a routine basis in a clinical
5 environment.

Indirect phenotyping can be defined as assigning a phenotype based on non-functional measurements. These non-functional measurements include genotyping, haplotyping, gene expression and protein expression analysis. The
10 patent application, WO 00/63683 provides an extensive description of various methods developed to perform the aforementioned analysis.

Genotyping is performed by analyzing the genetic sequence of a gene coding for a specific enzyme by a
15 polymerase chain reaction assay (PCR) or a PCR with a restriction fragment length polymorphism assay (PCR-RFLP). The gene is examined for the presence of genetic mutations that can be linked to increased or decreased enzyme levels or activity, which in turn result in a specific phenotype,
20 i.e. a slow metabolizer vs. a fast metabolizer. The genotype is a theoretical measurement of what an individual's phenotype should be. Haplotyping is an extension of genotyping in which the genotype of different gene alleles are considered. For example if a person had
25 one wild type (wt) gene sequence and one mutant (mt) gene sequence, the individual would have a wt/mt haplotype. Gene expression and protein expression analysis is defined as the measurement of mRNA/cDNA and protein levels respectively.

Indirect phenotyping may be limited by several factors that can result in an alteration in the theoretical phenotype. For example it has been well established that genotype does not always correlate with phenotype, likewise gene expression does not always correlate with protein expression, and protein expression does not always correlate with protein function. Indirect phenotyping fails to account for many factors that affect protein function including but not limited to post-translational protein modification, polypharmacy, and exposure to inducers or inhibitors. Furthermore, other limitations include the potential complexity of performing a complete genotyping. The mutation sequence must first be identified before they can be examined in a genotyping assay. Subsequent to identification, the mutation must be linked to a definitive effect on phenotype. For some enzymes, there appear to be very few mutations and those found have been well characterized, while for other enzymes multiple mutations are present with new mutations being found regularly (e.g. CYP2D6 has over 53 mutations and 48 allelic variants). Therefore, while genotyping for CYP2C19 might be performed with relatively few measurements, a complete and accurate genotyping of CYP2D6 would be complex and require multiple measurements.

Indirect phenotyping suffers from complexity and the direct phenotyping techniques are not easily accessible to clinical settings,

Physicians routinely prescribe treatment regimes without knowledge of an individual's metabolic capability (phenotype) or genotype for metabolism. Accordingly, a

trial and error treatment regime is initiated, often at the expense of severe side effects and loss of valuable treatment time.

5 The need for a method to predict an individual's response to a drug therapy (both efficacy of therapy and occurrence of side effects) has been recognized by many in the field. The importance of drug metabolizing can be explained as follows. If inhibition of a particular system leads to toxicity, then low gene or protein expression of components of this system might be used to identify individuals with high risk of toxicity. Likewise those individual's with high expression levels would be considered to be at low risk. However, if the individual classified as a low risk individual, also has low metabolism of the drug, then the drug will remain in the system much longer and may have the time to eliminate the function of the system which as a result leads to toxicity. Conversely, if an individual has low system activity but is also a rapid drug metabolizer, than it is possible that there will not be sufficient drug present at any given point to induce toxicity by inhibiting the system. Therefore, the knowledge of an individual's drug metabolizing capabilities is an essential component of individualized drug therapy.

25 The ability to rapidly and accurately identify multiple metabolic phenotypic determinants on an individual basis would provide a physician with valuable individual-specific information that could be readily applied in selecting a safe and effective treatment regime for that individual. Similarly, knowledge of multi-

determinant metabolic phenotypics would also find valuable application in research and drug development. In particular, individual phenotypes could be identified prior to a drug treatment trial. Moreover, knowledge of
5 multi-determinant metabolic phenotypes would have applications in the development of new drugs, so-called rational drug design.

SUMMARY OF THE INVENTION

10 One aim of the present invention is to provide a method for the individualization of treatment with a class of N-(aryl substituted)-naphthalidimide compounds.

Another aim of the present invention is to provide a method for the individualization of treatment with the
15 drug amonafide.

Another aim of the present invention is to provide a method of using a multi-determinant metabolic phenotype to individualize a treatment regimen for a class of N-(aryl substituted)-naphthalidimide compounds for an
20 individual, wherein the multi-determinant metabolic phenotype of the individual is determined; and a safe and therapeutically effective dose of the class of N-(aryl substituted)-naphthalidimide compounds is determined and/or selected based on the multi-determinant metabolic
25 phenotype of the individual.

Another aim of the present invention is to provide a method of using a multi-determinant metabolic phenotype to individualize a treatment regimen for amonafide for an individual, wherein the multi-determinant metabolic
30 phenotype of the individual is determined; and a safe and

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therapeutically effective dose of amonafide is determined and/or selected based on the multi-determinant metabolic phenotype of the individual.

Another aim of the present invention is to provide a
5 method of treating an individual having a condition treatable with a class of N-(aryl substituted)-naphthalidimide compounds, said method comprising: determining a multi-determinant metabolic phenotype of the individual; and administering a safe and therapeutically
10 effective dose of the class of N-(aryl substituted)-naphthalidimide compounds to the individual, wherein the dose has been determined based on a metabolic profile of the individual corresponding to the individual's metabolic phenotype for the class of N-(aryl substituted)-
15 naphthalidimide compounds as represented by the multi-determinant metabolic phenotype.

Another aim of the present invention is to provide a method of treating an individual having a condition treatable with amonafide with amonafide, the method
20 comprising: determining a multi-determinant metabolic phenotype of the individual; and administering a safe and therapeutically effective dose of amonafide to the individual, wherein the dose has been determined based on a metabolic profile of the individual corresponding to the
25 individual's metabolic phenotype for amonafide as represented by the multi-determinant metabolic phenotype.

Another aim of the present invention is to provide an assay system for detecting the presence of enzyme-specific metabolites in a biological sample, obtained from an
30 individual treated with a known amount of at least one

probe substrate for a class of N-(aryl substituted)-naphthalidimide compounds, specific for metabolic pathways of the metabolites, the assay comprising: means for receiving the biological sample, including a plurality of
5 affinity complexation agents contained therein; means for detecting presence of the enzyme-specific metabolites bound to the affinity complexation agents; and means for quantifying ratios of the metabolites to provide corresponding phenotypic determinants; wherein the
10 phenotypic determinants provide a metabolic phenotypic profile of the individual.

Another aim of the present invention is to provide an assay system for detecting the presence of enzyme-specific metabolites in a biological sample, obtained from an
15 individual treated with a known amount of at least one probe substrate for amonafide, specific for metabolic pathways of the metabolites, the assay comprising: means for receiving the biological sample, including a plurality of affinity complexation agents contained therein; means
20 for detecting presence of the enzyme-specific metabolites bound to the affinity complexation agents; and means for quantifying ratios of the metabolites to provide corresponding phenotypic determinants; wherein the phenotypic determinants provide a metabolic phenotypic
25 profile of the individual.

Another aim of the present invention is to provide a method of using an enzyme-specific assay for the individualization of treatment with a class of N-(aryl substituted)-naphthalidimide compounds, which comprises:
30 conducting the assay to identify phenotypic determinants

in a biological sample obtained from an individual treated with a probe substrate for the class of N-(aryl substituted)-naphthalidimide compounds; determining a rate of drug metabolism according to the determinants; and
5 determining and/or selecting a safe and therapeutically effective dose of the class of N-(aryl substituted)-naphthalidimide compounds for the individual based on the metabolic rate.

Another aim of the present invention is to provide
10 a method of using an enzyme-specific assay for the individualization of treatment with amonafide, which comprises: conducting the assay to identify phenotypic determinants in a biological sample obtained from an individual treated with a probe substrate for amonafide;
15 determining a rate of drug metabolism according to the determinants; and determining and/or selecting a safe and therapeutically effective dose of amonafide for the individual based on the metabolic rate.

Another aim of the present invention is to provide
20 a method of screening a plurality of individuals for treatment with a class of N-(aryl substituted)-naphthalidimide compounds, the method comprising: genotyping the individuals to identify individuals lacking at least one allelic variation known to prompt toxicity of
25 the class of N-(aryl substituted)-naphthalidimide compounds; and selecting individuals having a metabolic phenotype characterized as effective for metabolizing the class of N-(aryl substituted)-naphthalidimide compounds.

Another aim of the present invention is to provide
30 a method of screening a plurality of individuals for

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treatment with amonafide, the method comprising:
genotyping the individuals to identify individuals lacking
at least one allelic variation known to prompt toxicity of
amonafide; and selecting individuals having a metabolic
5 phenoytpe characterized as effective for metabolizing
amonafide.

A method of screening a plurality of individuals
for participation in a drug treatment trial assessing the
therapeutic effect of a candidate drug treatment, said
10 method comprising: genotyping each of the individuals to
identify individuals lacking at least one allelic
variation known to prompt the toxicity of the drug; and
characterizing a multi-determinant metabolic phenotype of
the identified individuals of the previous step to
15 determine each individual's ability to metabolize the
drug.

Another aim of the invention is to provide a
method for determining NAT2-specific phenotypic
determinants of an individual using a non-toxic probe
20 drug(s) to predict his/her predisposed metabolic capacity
for the drug amonafide. Thus, providing valuable
information about an individual's ability to metabolize
the identified drug and predict the likelihood of a toxic
response thereto. In doing so, the present invention
25 could be readily employed to screen individuals for their
metabolic capacity to metabolize amonafide and determine a
corresponding phenotype-specific dosage regime.

Accordingly, another aim of the present invention
is to provide a method for selecting an individual
30 treatment regimen for the drug amonafide.

Yet another aim of the present invention is to provide a method for selecting candidates for clinical treatment trials for Amonafide treatment.

Still another aim of the present invention is to
5 provide a method of using multi-determinant phenotyping for the individualization of treatment with amonafide.

In accordance with one aspect of the present invention, there is provide a method of individualizing treatment with a drug, wherein a drug dosage is determined
10 on an individual basis; said method comprising:
a) phenotyping said individual to determine his/her ability to metabolize said drug; and b) calculating an effective amount of said drug to prescribe to said individual corresponding to a rate of drug metabolism
15 specific to said individual.

In accordance with another aspect of the present invention, there is provided a method of using a NAT2-specific ELISA (Enzyme-Linked Immunosorbent Assay) for the individualization of treatment with amonafide, said method
20 comprising: a) conducting said ELISA to identify NAT2-specific determinants in a biological sample obtained from an individual treated with a probe substrate; b) calculating a rate of drug metabolism according to at least said NAT2-specific determinants; and c) determining
25 an individual dosage of amonafide corresponding to said rate of drug metabolism, wherein said rate of drug metabolism corresponds to the rate of metabolism of said probe substrate and is indicative of the rate of metabolism of amonafide in said individual.

In accordance with a further aspect of the present invention, there is provided a method of using an ELISA (Enzyme-Linked Immunosorbent Assay) specific to a plurality of phenotypic determinants and including phenotypic determinants of NAT2 and CYP1A2, for the individualization of treatment with amonafide, said method comprising: a) conducting said ELISA to identify said plurality of phenotypic determinants in a biological sample obtained from an individual treated with a probe substrate; b) calculating a rate of drug metabolism according to at least NAT2- and CYP1A2- specific determinants; and c) determining an individual dosage of amonafide corresponding to said rate of drug metabolism, wherein said rate of drug metabolism is indicative of the rate of metabolism of amonafide in said individual.

In accordance with yet another aspect of the present invention, there is provided a method of selectively treating individuals with amonafide, said method comprising: a) genotyping individuals to identify those individuals having at least one allelic variation known to prompt the toxicity of amonafide, and removing those individuals from a candidate treatment group; b) phenotyping those individuals identified as lacking said at least one allelic variation to determine each individual's ability to metabolize amonafide; and c) calculating an effective amount of said drug to prescribe to each of said individuals lacking said at least one allelic variation, said effective amount corresponding to a individual-specific rate of drug metabolism as determined by phenotypic determinants

specific for at least NAT2 enzyme, wherein each of said individuals lacking said at least one allelic variation is treated with an individually determined dosage of Amonafide.

5 In accordance with yet a further aspect of the invention, there is provided a method of screening candidates for participation in a drug treatment trial, said method comprising: a) genotyping each candidate to identify at least one allelic variation known to prompt
10 the toxicity of said drug, and removing those candidates from a trial group; b) phenotyping each candidate identified as lacking said at least one allelic variation to determine each candidate's ability to metabolize said drug; and c) selecting those candidates identified as
15 having an effective rate of metabolism for the drug for said trial, wherein said effective rate of metabolism is determined on the basis of at least phenotypic determinants specific for at least one enzyme known to metabolize the drug.

20 In accordance with still a further aspect of the invention, there is provided a method of selectively treating an individual with amonafide, said method comprising: a) detecting determinant-specific metabolites in a biological sample of an individual identified as a
25 having a condition treatable with amonafide, after said individual has been treatment with a probe substrate for amonafide; b) characterizing at least one phenotypic determinant based on amounts of said determinant-specific metabolites in said sample; c) determining if said at
30 least one phenotypic determinant corresponds to a

metabolic phenotype that qualifies as effectively
metabolizing amonafide; and d) if said individual
qualifies for amonafide treatment, determining an
individual dose specific to said individual's rate of
5 metabolism as calculated according to said at least one
phenotypic determinant.

For the purpose of the present invention the following
terms are defined below.

The term "phenotypic determinant" is intended to
10 mean a qualitative or quantitative indicator of an enzyme-
specific capacity of an individual.

The term "individualization" as it appears herein
with respect to therapy is intended to mean a therapy
having specificity to at least an individual's phenotype
15 as calculated according to a predetermined formula on an
individual basis.

The term "biological sample" is intended to mean a
sample obtained from a biological entity and includes, but
is not to be limited to, any one of the following: tissue,
20 cerebrospinal fluid, plasma, serum, saliva, blood, nasal
mucosa, urine, synovial fluid, microcapillary
microdialysis and breath.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Fig. 1 illustrates the basic structure of N-(aryl
substituted)-naphthalidimides according to an embodiment
of the present invention;

Fig. 2 illustrates metabolites of the NAT2 enzymatic pathway according to an embodiment of the present invention;

Fig. 3 illustrates metabolites of the CYP1A2 enzymatic pathway according to another embodiment of the present invention;

Fig. 4 illustrates metabolites of the CYP3A4 enzymatic pathway according to another embodiment of the present invention;

Fig. 5 illustrates metabolites of the NAT1 enzymatic pathway according to another embodiment of the present invention;

Fig. 6 illustrates metabolites of the CYP2A6 enzymatic pathway according to another embodiment of the present invention;

Fig. 7 illustrates metabolites of the CYP2C19 enzymatic pathway according to another embodiment of the present invention;

Fig. 8 illustrates metabolites of the CYP2C9 enzymatic pathway according to another embodiment of the present invention;

Fig. 9 illustrates metabolites of the CYP2D6 enzymatic pathway according to another embodiment of the present invention;

Fig. 10 illustrates metabolites of the CYP2E1 enzymatic pathway according to another embodiment of the present invention;

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Fig. 11 illustrates the scheme of the general immunosensor design depicting the intimate integration of immunological recognition at the solid-state surface and the signal transduction;

5 Fig. 12 illustrates the principle of SPR technology;

Fig. 13 illustrates a TSM immunosensor device;

Fig. 14 illustrates the synthetic routes for the production of AAMU and 1X derivatives used in accordance
10 with one embodiment of the present invention;

Figs. 15 to 18 show other AAMU and 1X derivatives which can be used for raising antibodies in accordance with another embodiment of the present invention;

Fig. 19 illustrates the absorbance competitive
15 antigen ELISA curves of AAMU-Ab and 1X-Ab in accordance with one embodiment of the present invention;

Fig. 20 is a histogram of molar ratio of AAMU/1X;

Fig. 21 illustrates the synthetic routes for the production of caffeine and 1,7-dimethylxanthine deriva-
20 tives for CYP1A2 phenotyping in accordance with one embodiment of the present invention;

Fig. 22 illustrates the synthetic routes for the production of caffeine and 1,7-dimethyluric acid deriva-
25 tives for CYP1A2 phenotyping in accordance with one embodiment of the present invention;

Fig. 23 illustrates an array of microwell plates as employed in accordance with another embodiment of the present invention;

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Fig. 24 illustrates an ELISA array in accordance with an embodiment of the present invention;

Fig. 25 illustrates an example of the configuration of a microwell plate in accordance with another embodiment of the present invention;

Fig. 26 illustrates an ELISA detection system in accordance with another embodiment of the present invention;

Fig. 28 illustrates individualized dosing schemes for direct vs. indirect phenotyping in accordance with yet another embodiment of the present invention;

Fig. 29 illustrates a nomogram for the determination of body surface area in accordance with yet another embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of metabolic phenotyping for the individualization of drug treatment. In particular the present invention relates to the individualization of treatment with a class of compounds termed N-(Aryl substituted)-naphthalidimides. More particularly, the present invention relates to the individualization of treatment with the drug amonafide based on a phenotypic characterization of an individual's capacity to metabolize the drug. Amonafide is known to be metabolized by the NAT2 and CYP1A2 metabolic pathways. The present invention provides a method for quickly and accurately determining phenotypic determinants for the NAT2 and CYP1A2 pathway that can be used to characterize an individual's specific phenotypes. Further, the present

invention provides a method for determining multiple phenotypic determinants that can be used to characterize a phenotypic profile of an individual that will exemplified that individual's ability to metabolize a given drug.

5 Although most drugs are metabolized by a primary enzymatic pathway, it is often the case that an individual's phenotype for that pathway will be influence (e.g. inhibited) by other enzymatic events occurring at any given time. As a result, it may be preferred to
10 characterize a phenotypic profile of an individual prior to selecting a corresponding drug treatment regime. Knowledge of an individual's metabolic phenotype may be applied clinically in determining a phenotype-specific dose based on the individual's capacity to metabolism a
15 given drug. Other factors representing an individual's capacity to metabolize a drug may also find application in the present invention, together with a phenotypic profile for obtaining individualization of therapy.

Accordingly, the present invention will be
20 exemplified in accordance with methods of determining phenotypic determinants for NAT2. The determination of metabolic determinants for NAT2 may be performed as a single determination or in combination with methods of determining a phenotypic profile for any other drug
25 metabolizing enzymes including at least one of the following enzymes: NAT1, CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19, UGT, GST, ST. The metabolites of suitable probe substrates for some of these enzymes are illustrated in Figs. 1-9. These enzymes are involved in
30 the metabolism of a large number of drugs, and as a result

have important implications in the outcome of individual drug treatment regimes, and hence, clinical trial studies. These enzymes and their corresponding phenotypic determinants as described herein are provided as a
5 representative example of determinants for the purposes of exemplifying the multi-determinant metabolic phenotyping of the present invention. However, the present invention is not limited thereto.

The present invention provides the ability to
10 identify multiple phenotypic determinants of these enzymatic pathways for use in the individualization of drug treatment. The present invention provides a method of individualized treatment of cancer, with a class of compounds termed N-(Aryl substituted)-naphthalidimides.
15 In particular, the present invention provides a method of individualized treatment of cancer, with the drug amonafide.

Many exemplars of the class of compounds termed N-(Aryl substituted)-naphthalidimides are described in the
20 following patents; US4614820; US4665071; US4594346; US499266; US4204063; and US4874863 and publications; Brana et al. 1997, J Med Chem 40, 449-454; Brana et al. 1999, J Med Chem 42, 5482-5486; Gamage et al. 2000 J Med Chem 44, 1407-1415. The basic structure of a member of the N-(Aryl
25 substituted)-naphthalidimides class of compounds is illustrated in Figure 1. The present invention covers individualization of therapy for all compounds described in these patents as well as all other derivatives of the basic structure including but not limited to arylamino-

maphthalidimides, arylhydroxy-naphthalidimides and all acyl derivatives thereof. Additionally, the present invention covers individualization of all pharmaceutically acceptable salts or composition of said compounds.

5 Amonafide (Benzisoquinolinedione, NSC 308847) is an imide derivative of naphthalic acid. Naphthalic acid is of interest because of its inhibitory effect on cellular replication. The cytotoxic activity of naphthalic acid is maximal when its side chain is composed of two methylene groups with terminal nitrogen. Amonafide meets this requirement and has demonstrated significant activity against the L1210 and p388 leukemia cells lines as well as B16 melanoma and M5076 sarcoma cell line. Amonafide is a site-specific intercalating agent and a topoisomerase II inhibitor (Warning et al. (1979) Nucl. Acid Res. 7: 217-230; Hsiang et al. (1989) Mol. Pharmacol. 36: 371-376).

Preclinical toxicity studies in mice, dogs, and rats indicate that amonafide produces reversible hematopoietic, gastrointestinal, renal and hepatic toxicity. In addition, signs indicative of neurotoxicity were seen in dogs in both single-bolus and daily for 5 days schedules at the highest doses administered.

Clinical Pharmacology

25 N-acetylation is an important pathway for the metabolism of aromatic and hydrazine drugs (Weber and Hein (1985) Pharmacol. Rev. 37: 25-79). Examples of commonly used drugs metabolized by N-acetylation include

procainamide, dapsone, aminogluthethimide, isoniazid, hydralazine, sulfasalazine and caffeine. The acetylator phenotype is inherited autosomally, with the allele for the slow phenotype (homozygous) predominating in the North American population. The estimate incidence of slow acetylators is 60%, with the remaining 40% considered either rapid or indeterminate acetylators. The true "rapid" acetylator is a homozygote (5% of population) for the less common rapid allele, whereas the indeterminate phenotype (called rapid by some authors) is a heterozygote, comprising approximately 35% of the population. There are significant ethnic and geographic correlates with acetylator phenotype (Weber and Hein, *supra*; Clark (1985) *Drugs* 29: 342-375). In particular, the slow acetylator phenotype is relatively uncommon (<10%) in Orientals and Eskimos, whereas Mid-Eastern populations are comprised almost exclusively of slow acetylators.

Preliminary human pharmacology studies have reported that the volume of distribution of amonafide is large and that it is highly bound to body tissues. Amonafide is extensively metabolized and metabolites have been detected in both plasma and urine. Amonafide is metabolized by both N-acetylation and N-oxidation (Felder *et al.* (1987) *Drug Metab. Dispos.* 15: 773-778). It has been reported that N-acetyl-amonafide is approximately equipotent to the parent drug, whereas N-oxide-amonafide is essentially inactive (Felder *et al.*, *supra*). The degree of acetylation may account for the variability in pharmacokinetics and in the toxicity seen at the various

doses tested, since N-acetyl-amonafide is cytotoxic (Felder *et al.*, *supra*).

Phase I studies were performed at the UTSA (University of Texas at San Antonio) (Saez *et al.* (1989) J. Clin. Oncol. 7: 1351-1358), OSU (Ohio State University) (Leiby *et al.* (1988) Proc. Am. Assoc. Cancer Res. 29:278) and MDA (M. D. Anderson) (Legha *et al.* (1987) Cancer Treat. Rep. 71: 1165-1169). When given as a single bolus, the maximum tolerated dose (MTD) was 800 mg/m². The dose-limiting toxicity was myelosuppression (Saez *et al.*, *supra*). When daily times five dose was given every 21 days, the MTD was 400 mg/m² (Legha *et al.*, *supra*) or 250 mg/m² (Leiby *et al.*, *supra*). Again myelosuppression was the dose-limiting toxicity. Other reported toxicities were mild to moderate nausea, vomiting and alopecia. All three centers reported acute toxicity with rapid amonafide infusion. This constituted of local inflammatory reactions, diaphoresis, flushing, tinnitus, headache and/or dizziness. Increasing the duration of infusion to 1 hour minimized these effects. Regarding the different regimens, no schedule dependency was noted. The bolus dose MTD was different in two studies. The UTSA group (Saez *et al.*, *supra*) showed a highly variable and individualized hematotoxicity at sub-MTD doses, although they did reach a MTD of 800 mg/m². The OSU study (Leiby *et al.*, *supra*) reported an MTD of 1125 mg/m² but noted considerable and variable toxicity. The same group also compared bolus dose (1125 mg/m²) to a 5-day schedule at 288 mg/m² and concluded that the 5-day schedule was preferred since more drug could be given (Leiby *et al.*, *supra*). In

another 5-day schedule, the MDA study (Legha et al., *supra*) found a higher MTD of 400 mg/m². The recommended Phase 2 doses were 200 mg/m² and 400 mg/m² (300-320 mg/m² in poor risk individuals).

5 From 1990 to 1998, 33 Phase 2 trials and 1 Phase 3 trial were conducted by different groups including the Gynecologic Oncology Group (GOG), the South Western Oncology Group (SWOG), the Eastern Cooperative Oncology Group (ECOG), the Cancer and Leukemia Group B (CALBG), and
10 the Illinois Cancer Center Trial. A total of 981 individuals received amonafide for a number of tumor types. For most studies, the dose and schedule of amonafide was 300 mg/m² daily for 5 days every 3 weeks.

 Three Phase 2 trials were conducted in metastatic
15 breast cancer, as first line chemotherapy: two were done with a dosage regimen of 800-900 mg/m² over 3 hours repeated every 4 weeks (Kornek et al. (1994) Eur. J. Cancer 30A(3): 398-400) and one with 300 mg/m² daily for 5 consecutive days every 3 weeks for 4 cycles, followed by
20 CAF (Costanza et al. (1995) Clin Cancer Res. 1: 699-704). In the only Phase 3 trial, individuals received 300 mg/m² 4 times a day for 5 consecutive days every 3 weeks for 4 cycles, followed by CAF (Costanza et al. (1999) J. Clin. Oncol. 17(5): 1397-1406). Amonafide was found to be an
25 active compound for the treatment of individuals with advanced breast cancer, with a response rate ranging from 18% to 50% in individuals with grade 3 or 4 thrombocytopenia.

NAT2 enzyme polymorphism seems to play an important role in the metabolism of amonafide. In 1991, Ratain et al. (Ratain et al. (1991) Clin. Pharmacol. Ther. 50(5):573-579) investigated a phenotyping procedure for acetylation using caffeine as a probe in cancer individuals treated with amonafide. Slow and fast acetylators of both caffeine and amonafide were identified. The production of the acetylated metabolite was one of the major determinants of myelosuppression, since fast acetylators had greater toxicity than slow acetylators (Ratain et al. (1991) Clin. Pharmacol. Ther. 50(5):573-579). However, the area under the plasma concentration-time curve of amonafide was significantly greater in fast acetylators, who would be expected to have a higher clearance and a lower area under the plasma concentration time-curve. This appeared to be an unusual finding compared to other drugs metabolized by N-acetylation. For most drugs, slow acetylators are at greater risk of adverse events. The unexpected behavior of amonafide was due to the inhibition of amonafide oxidation by N-acetyl-amonafide, since *in vitro* studies demonstrated that amonafide is a substrate for CYP1A2 and amonafide's oxidation is inhibited by its acetylated metabolite (Ratain et al. (1995) J. Clin. Oncol. 13: 741-747). Based on the acetylator phenotyping, new recommended doses were proposed for slow acetylators (375 mg/m²/day) and fast acetylators (250 mg/m²/day) (Ratain et al. (1993) Cancer Res. 53: 2304-2308). A fixed dose of amonafide of 300 mg/m² was considered inappropriate for all individuals, as fast phenotypes would be expected to

experience grade 4 toxicity and slow phenotypes may be significantly under dosed (Ratain et al. (1991) Clin. Pharmacol. Ther. 50(5):573-579). Since there was still significant inter-individual variability in toxicity at these new dose levels, a subsequent study attempted to develop pharmacodynamic models to individualize amonafide's dosing. The optimal model was defined by acetylator phenotype, pretreatment white blood cell count (WBC) and gender (Ratain et al. (1996) Pharmacogenetics 6: 93-101).

In contrast, an embodiment of the present invention will provide for the individualization of dosing with amonafide in direct relation to the quantitative value of an individual's molar ratio calculated during NAT2 phenotyping. Accordingly, dosing can be individualized, as opposed to categorized, to account for an individual's specific capacity to metabolize the drug. Additionally, the present invention allows for the monitoring of multiple phenotypic determinants, in particular CYP1A2 function. The analysis of multiple phenotypic determinants may play an essential role in the individualization as a result of the role of both N-acetylation (NAT2) and n-oxidation (CYP1A2) in the metabolism of amonafide.

In addition, the present invention describes the use of indirect phenotyping to identify individuals with a particular genotype, which is associated with extremely high risks of amonafide toxicity. According to this embodiment of the present invention, those individuals

without the "high risk" genotype will be phenotyped and dosed according to their individual molar ratio. The high risk individuals will not be prescribed amonafide. By employing genotyping in combination with phenotyping to
5 screen individual's for treatment with amonafide, those individuals found to be carrier of a high risk genotype can be eliminated as candidates for such treatment without the necessity of phenotyping.

The integration of phenotyping tests into the drug
10 development process will allow for a decreased number of individuals participating in a drug treatment testing trial, as individual screening using phenotyping can be conducted prior to the trial to select those individuals displaying the capability to metabolize the drug of
15 interest safely and effectively. In particular, those individuals identified as being metabolically incompatible with the drug treatment trial can be screened out before undergoing treatment with the drug. This aspect of the present invention will provide a means to selectively
20 treat only those individuals identified as having the ability to safely metabolize the drug. In addition, the decrease in individual number will result in decreased costs and will allow the drug to reach the market faster. In addition, the clinical use of a phenotypic screening
25 method will provide the ability to individualize treatments according to phenotypic profiles. In particular, dose specific determinations corresponding to a calculated rate of metabolism for that drug phenotype will be possible on an individual basis.

Pre-trial screening would involve the phenotyping of all individuals prior to inclusion in the trial. The phenotype status could then be used to identify those individuals at high risk for (Severe Adverse Events) SAE's and ensure that they were not included in the trial. The remaining individuals would then be treated with drug doses individualized in correlation to their level of NAT2 activity. The individualized dose would ensure that the individuals were receiving a safe efficacious treatment, corresponding to their ability to safely metabolize the drug. Similarly, according to the present invention, individualized treatment will find application in the clinical environment where drug treatment dosages will be individualized according to an individual's phenotypic profile or calculated rate of metabolism.

According to the present invention, phenotypic determinants for one or more of the following enzymes may be characterized to provide a phenotypic profile on an individual basis:

20 NAT2

Polymorphism

Individuals are genetically polymorphic in their rate of N-acetylation of drugs via the N-acetyltransferase (NAT2) pathway (Meyer, U.A. (1994) Proc. Natl. Acad. Sci. USA, 91:1983-1984). Two major metabolic phenotypes can be distinguished: fast and slow N-acetylators. Drugs that are individual to N-acetylation polymorphism include sulfonamides (sulfamethazine), antidepressants

(phenelzine), antiarrhythmics (procainamide), and antihypertensives (hydrazine). Some adverse therapeutic consequences of the acetylator phenotype are peripheral neuropathy and hepatitis. In an opposite manner, the N-acetylation of procainamide produces a therapeutically active metabolite with reduced toxicity. N-acetylation polymorphism has also been linked to the detoxification pathway of some environmental carcinogenic arylamines and there is a higher frequency of bladder cancers among chemical dye workers who are slow N-acetylators.

The NAT2 gene is polymorphic, there have been 9 mutations detected and 14 mutant alleles. Six mutant alleles are responsible for 99% of Caucasian slow acetylators (NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6A, NAT2*7B, and NAT2*13). The NAT2*4 allele is the wild-type allele.

Inter Ethnic Differences

The frequencies of PM (poor metabolizer) and EM (extensive metabolizers) (autosomal recessive trait) show considerable inter ethnic differences for the N-acetylation polymorphism. In Caucasians, the frequencies are approximately 60 and 40%, respectively, while in Orientals, they are 20 and 80%, respectively (Meyer, U.A. (1994) Proc. Natl. Acad. Sci. USA, 91:1983-1984). It is reasonable that, in drug metabolism studies, each ethnic group is studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Direct Phenotyping - Phenotypic Determinants of NAT2

Different probe substrates can be used to determine the NAT2 phenotype. In accordance with the present invention a suitable probe substrate is, without
5 limitation caffeine. Caffeine is widely consumed and relatively safe. A phenotype may be generally determined from ratios of the caffeine metabolites 5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU) and 1-methylxanthine (1X) present in
10 urine samples of an individual collected after drinking coffee. The structures of these metabolites are illustrated in Fig. 9. The ratio of these metabolites provides a determination of an individual's N-acetylation (NAT2) phenotype.

15
$$\text{AAMU (or AFMU) / 1X}$$

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the acetylation phenotype of the individual as follows. Individuals with a ratio less than 1.80 are slow
20 acetylators.

Indirect Phenotyping (Genotyping)

An example of NAT2 genotyping involves the amplification of a 547 bp fragment which includes the 5' of the 6 mutant alleles which are responsible for 99% of
25 Caucasian slow acetylators. Analysis of these 5 alleles and the wt allele can be performed by examining 4 mutations (Smith CAD et al. J Med Genet (1997) 34:758-760).

The PCR amplification is performed with the following primers:

5'-GCTGGGTCTGGAAGCTCCTC-3'

5'-TTGGGTGATACATACACAAGGG-3'

5 The analysis of this fragment with 4 restriction digestion enzymes allows the detection of 6 alleles (NAT2*4 (wt) and the mutants NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6 and NAT2*7). Each of the 6 alleles have distinct combinations of the mutations and as each mutation alters
10 a specific restriction digestion enzyme site (*KpnI*, *DdeI*, *TaqI* or *BamHI*), the performance of 4 separate digestions of the 547 bp fragment will allow the identification of the different alleles.

CYP1A2

15 CYP1A2 constitutes 15% of the total CYP 450 enzymes in the human liver.

Polymorphism

CYP1A2 may be polymorphic although it remains to be established firmly. To date no mutant alleles have
20 been identified. Three metabolic phenotypes can be distinguished: rapid, intermediate and slow metabolizers. CYP1A2 metabolizes several drugs and dietary constituents including resiquimod, imiquimod, tacrine, acetaminophen, anti pyrine, 17 β -estradiol, caffeine, cloipramine,
25 clozapine, flutamide (antiandrogenic), imipramine, paracetamol, phenacetin, tacrine and theophylline.

In addition, CYP1A2 activates environmental pro-carcinogens, especially heterocyclic amines and aromatic

amines. In one study it has been shown that individuals who are fast N-acetylators and have high CYP1A2 activity are at a greater risk for colorectal cancer (35% of cases vs. 16% of controls, OR=2.79 (P=0.00-2)).

5 Induction and Inhibition

CYP1A2 is induced by a number of drugs and environmental factors such as omeprazole, lansoprazole, polyaromatic hydrocarbons and cigarette smoke. CYP1A2 is inhibited by oral contraceptives, ketoconazole, 10 α -naphthoflavone, fluvoxamine (serotonin uptake inhibitor), and furafylline.

Inter Ethnic Differences

The activity of CYP1A2 varies broadly (60 to 70 fold) in a given population. Slow, intermediate and rapid 15 CYP1A2 phenotypes have been distinguished. The proportion of these three CYP1A2 phenotypes varied between ethnic groups and countries: % of intermediates: 50, 70, 60, >95, 60, 20 in U.S.A., African-American, China, Japan, Italy and Australia, respectively. It is reasonable that, 20 in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Theophylline

25 A classical example of the need for phenotyping in drug dosing is the case of theophylline. Theophylline is used in the treatment of asthma. However, theophylline toxicity continues to be a common clinical problem, and

involves life-threatening cardiovascular and neurological toxicity. Theophylline is cleared from the body via the CYP1A2 metabolizing system. Inhibition of CYP1A2 by quinolone antibiotic agents or serotonin reuptake inhibitors may result in theophylline toxicity. For these reasons, the utility of a reliable phenotyping test for CYP1A2 is evident.

Direct Phenotypic Determinants of CYP1A2

Different probe substrates can be used to determine the CYP1A2 phenotype (caffeine, theophylline). In accordance with the present invention suitable probe substrates include without limitation, caffeine, theophylline or acetaminophen.

Of these caffeine is the preferred probe substrate. Caffeine is widely consumed and relatively safe. The structure of caffeine and its metabolites 1,7-dimethylxanthine (1,7 DMX) and 1,7-dimethyluric acid (1,7 DMU) are illustrated in Fig. 3.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the CYP1A2 phenotype of the individual as follows:

$$\frac{1,7\text{-dimethylxanthine (1,7 DMX)} + 1,7\text{-dimethyluric acid (1,7 DMU)}}{\text{caffeine}}$$

Molar ratios of 4 and 12 separate slow, intermediate and fast CYP1A2 metabolizers, respectively (Butler et al. (1992) Pharmacogenetics 2:116-117).

Indirect Phenotypic Determinants of CYP1A2 (Genotyping)

To date no mutant alleles have been identified for the CYP1A2 gene. Therefore, indirect phenotyping is not currently possible for CYP1A2.

5 CYP3A4

The CYP 3A family constitutes approximately 25% of the total CYP 450 enzymes in the human liver.

Polymorphism

10 A large degree of inter-individual variability in the expression of the CYP3A4 isoenzymes has been shown in the human liver (>20 fold). However, the activity of CYP3A4 metabolism is distributed unimodally and as a result, there are currently no categorical classifications for distinct subsets of this population. Further, there is currently no evidence of a common allelic variant in the coding region of the gene. Recently, a rare allelic variant was identified in exon 7 (CYP3A4*2). Limited data suggested that this mutation may result in altered substrate dependent kinetics compared with the wt CYP3A gene. It has been considered that the large inter-individual variability in the activity of CYP3A may reflect differences in transcriptional regulation. Another allelic variant in the 5'-flanking region of CYP3A has been identified (CYP3A4*1B) that involves an A→G transition at position -290 from the transcriptional initiation site. It has been speculated that this nucleotide substitution may be associated with a reduced level of CYP3A activity. Ongoing studies are

20

25

investigating the existence of a common allelic variant linked to CYP3A4 activity.

CYP3A4 metabolizes several drugs and dietary constituents including delavirdine, indinavir, ritonavir, saquinavir, amprenavir, zidovudine (AZT), nelfinavir mesylate, efavirenz, nevirapine, imiquimod, resiquimod, donepezil, lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin, rosuvastatin, benzafibrate, clofibrate, fenofibrate, gemfibrozil, niacin, benzodiazepines, erythromycin, dextromethorphan dihydropyridines, cyclosporine, lidocaine, midazolam, nifedipine, and terfenadine.

In addition, CYP3A4 activates environmental pro-carcinogens especially N'-nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), 5-Methylchrysene, and 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products).

Induction and Inhibition

CYP3A4 is induced by a number of drugs including dexamethasone, phenobarbital, primidone and the antibiotic rifampicin. Conversely, CYP3A4 is inhibited by erythromycin, grapefruit juice, indinavir, ketoconazole, miconazole, quinine, and saquinavir.

Inter Ethnic Differences

Several studies have suggested that the activity of CYP3A4 varies between populations. Plasma levels of a CYP3A4 substrate drug after oral administration were reported to be twofold to threefold higher in Japanese,

Mexican, Southeast Asian and Nigerian Populations compared with white persons residing in various countries. In addition, the CYP3A4*1B allele has been reported to be more frequent in African-American populations as compared to European Americans or Chinese populations (66.7% vs. 4.2% vs. 0%, respectively). The rare CYP3A4*2 allele was found in 2.7% of a white population and was absent in the black and Chinese individuals. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

Due to the variability in CYP3A4 activity within the population it would be advantageous to be provided with a system and method for quickly and easily determining an individual's CYP3A4 metabolic phenotype prior to administering a CYP3A4-dependant treatment thereto. In particular, such a system and method are believed to have enormous benefit in the individualization of therapy, and in particular with respect to the individualization of therapy with many hyperlipidia agents, including HMG-CoA reductase inhibitors (statins), fibrates, bile acid sequestrants and nicotinic acid (niacin).

25 Cyclosporine

An example of the need for phenotyping in drug dosing is the case of cyclosporine in the treatment of organ transplant individuals. Cyclosporine is an immunosuppressant agent (drug) administered post

transplant to protect the new organ from being rejected. Plasma levels of this drug are critical as high levels lead to renal toxicity but low levels can lead to organ rejection. Cyclosporine is metabolized via the CYP3A4 system. Several studies have indicated the importance of monitoring CYP3A4 activity in maintaining an effective and safe cyclosporine dose. For these reasons, the utility of a reliable phenotyping test for CYP3A4 is evident.

Direct Phenotypic Determinants of CYP3A4

Different probe substrates can be used to determine the CYP3A4 phenotype (dapsone, testosterone, nifedipine, midazolam, erythromycin, dextromethorphan, cortisol). In accordance with the present invention, suitable probe substrates include without limitation, midazolam, dextromethorphan, erythromycin, dapsone, testosterone, nifedipine and cortisol.

Of these midazolam is the preferred probe substrate. The structures of midazolam and its hydroxylated metabolite, 1'-hydroxymidazolam are illustrated in Fig. 1. In accordance with the present invention, the molar ratio of midazolam and its metabolite is used to determine the CYP3A4 phenotype of the individual as follows:

$$\frac{1'\text{-hydroxymidazolam}}{\text{midazolam}}$$

An individual's ratio will be considered as indicative of CYP3A4 enzyme activity with a lower ratio indicating poorer metabolism and a higher ratio indicating more extensive metabolism. The activity of CYP3A4

metabolism is distributed unimodally and hence no antimode is present. The levels of CYP3A4 activity as determined by direct phenotyping will be used.

Indirect Phenotypic Determinants of CYP3A4 (Genotyping)

5 To date only two mutant alleles have been identified for the CYP3A4 gene (CYP3A4*1B and CYP3A4*2). Studies have been unable to correlate these mutations with the large inter-individual variation in CYP3A4 activity. Despite confirmation in this regard to date, the use of
10 indirect phenotyping is contemplated in accordance with the present invention. Ongoing studies continue to investigate this aspect of the present invention.

NAT1

15 The NAT1 enzyme catalyzes the N-acetylation of many compounds. It is expressed in the liver as well as in mononuclear leucocytes.

Polymorphism

20 The NAT1 gene was for a long time classified as monomorphic. However, it is now suggested that NAT1, like the other N-acetyltransferase gene (NAT2), is polymorphic. Studies have demonstrated the presence of one wild type allele (NAT1*4) and six mutant alleles (NAT1*3, NAT1*5, NAT1*10, NAT1*11, NAT1*14 and NAT1*17). NAT1 has two phenotypes: slow and rapid acetylators (e.g. NAT1*4 vs.
25 NAT1*10 genotypes respectively).

NAT1 metabolizes several drugs and dietary constituents including p-aminobenzoic acid, p-aminosalicylic acid, and dapsone.

In addition, NAT1 activates environmental pro-carcinogens, especially diaminobenzidine, N-hydroxy-4-aminobiphenyl, and heterocyclic aromatic amines (MeIQx and PhIP). In one study it has been shown that individuals
5 who have the NAT1*10 allele, and hence are rapid N-acetylators, are at a greater risk for colorectal cancer (OR=1.9; 95% CI=1.2-3.2), while in another study they have an increased risk for bladder cancer (metabolize benzidine).

10 Inter Ethnic Differences

The activity of NAT1 varies broadly in a given population. Slow, and rapid NAT1 phenotypes have been distinguished. The NAT1*10 genotype that is associated with rapid metabolic phenotype was monitored in three
15 different ethnic populations, Indian, Malaysian and Chinese. The frequency of NAT1*10 allele was 17%, 39% and 30%, respectively. The NAT1*4 genotype, associated with slow metabolizers, had a frequency in the same populations of 50%, 30% and 35%, respectively. Therefore, it is
20 reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Dapsone

25 A classical example of the need for phenotyping in drug dosing is the case of dapsone. Dapsone is used in the treatment of malaria and is being investigated for the treatment of *Pneumocystis carinii* pneumonia in AIDS individuals. Adverse effects include rash, anemia,

methemoglobinemia, agranulocytosis, and hepatic dysfunction. Dapsone is cleared from the body via the NAT1 metabolizing system. A study has shown a correlation between slow acetylation and increased adverse reactions to dapsone (46% vs. 17% for slow and fast acetylators, respectively). For these reasons, the utility of a reliable phenotyping test is evident.

Phenotypic Determinants of NAT1

Different probe substrates can be used to determine the NAT1 phenotype, such as (p-aminosalicylic acid (pASA), and p-aminobenzoic acid (pABA)). In accordance with the present invention suitable probe substrates include, with out limitation, p-aminosalicylic acid, and p-aminobenzoic acid.

Of these pASA is the preferred probe substrate. The structure of pASA and its acetylated metabolite p-acetylaminosalicylic acid are illustrated in Fig. 2.

In accordance with the present invention, the molar ratio of pASA and its acetylated metabolite is used to determine the NAT1 phenotype of the individual as follows:

$$\frac{\text{pASA}}{\text{pAcetyl-ASA}}$$

Indirect Phenotypic Determinants of NAT1 (Genotyping)

The NAT1 alleles NAT1*4 (wt) and the mutant NAT1*14 can be determined either by PCR-RFLP or allele specific PCR (Hickman, D. et al. (1998); Gut 42:402-409). The PCR-RFLP methodology requires the amplification of the

fragment of gene containing the A560G mutation. This is performed with the following primers:

5'-TCCTAGAAGACAGCAACGACC-3'

5'-GTGAAGCCCCACCAAACAG-3'

5 This PCR amplification produces a 175 bp fragment that is incubated with the *BsaI* restriction enzyme. The Nat1*4 allele is cleaved and produces a 155 bp fragment and a 20 bp fragment, while the mutant NAT1*14 is uncleaved.

10 The NAT1*14 allele is confirmed using an allele specific PCR, with the following primers:

5'-TCCTAGAAGACAGCAACGACC-3'

5'-GGCCATCTTTTAAAATACATTTT-3'

CYP2A6

15 CYP2A6 constitutes 4% of the total CYP 450 enzymes in the human liver. CYP2A6 is estimated as participating in 2.5% of drug metabolism.

Polymorphism

20 CYP2A6 is functionally polymorphic with two mutant alleles, CYP2A6*2 and CYP2A6*3, resulting in an inactive enzyme or the absence of the enzyme, respectively. Two metabolic phenotypes can be distinguished: poor and extensive metabolizers. CYP2A6 metabolizes several drugs including neuroleptic drugs and volatile anesthetics as
25 well as the natural compounds, coumarin, nicotine and aflatoxin B1.

In addition, CYP2A6 activates several components of tobacco smoke (e.g. NNK), as well as 6-aminochrysene. The role of activation of tobacco smoke and the metabolism of nicotine have suggested a role for CYP2A6 in the
5 development of smoking related cancers.

Induction and Inhibition

CYP2A6 is induced by barbiturates, antiepileptic drugs and corticosteroids.

Inter Ethnic Differences

10 CYP2A6 demonstrates marked inter-individual variability and has demonstrated ethnic related differences. The proportion of the two phenotypes varied between ethnic groups and countries: % of wt genotype (extensive metabolizers): 85, 76, 52, 83, 97.5 in Finnish,
15 English, Japanese, Taiwanese and African-American populations, respectively. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to
20 another.

Nicotine

An example of the need for phenotyping in drug dosing is in the delivery of nicotine, for a smoking cessation program. CYP2A6 is the primary means of
25 nicotine metabolism. Extensive CYP2A6 metabolizers will eliminate nicotine at a much higher rate. Identification of individuals with an increased CYP2A6 activity and hence increased nicotine metabolism may identify those

individuals that will require higher doses of nicotine at the onset of their attempt to quit smoking with the assistance of a nicotine delivery system. Alternatively, these individuals may benefit from non-nicotine delivery systems for assisting in quitting smoking.

Direct Phenotypic Determinants of CYP2A6

A probe substrate can be used to determine the CYP2A6 phenotype (coumarin). In accordance with the present invention suitable probe substrates include, without limitation, coumarin. The structure of coumarin and its metabolite 7-hydroxycoumarin are illustrated in Fig. 4.

In accordance with the present invention, the molar ratio of coumarin and its metabolite, 7-hydroxycoumarin is used to determine the CYP2A6 phenotype of the individual as follows:

$$\frac{\text{7-hydroxycoumarin}}{\text{coumarin}}$$

Indirect Phenotypic Determinants of CYP2A6 (genotyping)

Currently three alleles have been identified for the CYP2A6 gene, the wild type allele (CYP2A6*1) and two mutant alleles (CYP2A6*2, and CYP2A6*3). The wt allele codes for a fully functional enzyme. The CYP2A6*2 mutant allele codes for an inactive enzyme and the CYP2A6*3 allele does not produce any enzyme.

Determination of an individual genotype can be performed by a combined LA-PCR and PCR-RFLP procedure. In this procedure, specific oligonucleotide primers were used

to amplify the CYP2A6/7 gene. The amplified CYP2A6/7 gene is then used as the PCR template to amplify exons 3 and 4 using specific oligonucleotide primers to amplify a 544 bp fragment. This fragment is then digested with the *FspI* restriction enzyme and a 489 bp fragment re-isolated. This 489 bp fragment is then incubated with both *DdeI* and *XcmI*. The digestion patterns were determined by electrophoresis. The wildtype allele produces 330, 87 and 72 bp fragments, the CYP2A6*2 allele yields 189, 141, 87 and 72 bp fragments and the CYP2A6*3 allele yields 270, 87, 72, 60 bp fragments (Nakajima et al. (2000) Clin Pharmacol & Ther. 67(1):57-69).

Primers

CYP2A6/7 LA-PCR

5'-CCTCCCTTGCTGGCTGTGTCCCAAGCTAGGC-3'
5'-CGCCCCTTCCTTTCCGCCATCCTGCCCCCAG-3'

Exon 3/4 PCR

5'-GCGTGGTATTTCAGCAACGGG-3'
5'-TGCCCCGTGGAGGTTGACG-3'

CYP2C19

CYP2C19 accounts for about 2% of oxidative drug metabolism. CYP2C19 has been postulated as participating in ~8% of drug metabolism.

Polymorphism

Individuals are genetically polymorphic with respect to CYP2C19 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers.

Two genetic polymorphisms have been identified (CYP2C19*2 and CYP2C19*3) that together explain all of the Oriental poor metabolizers and about 83% of Caucasian poor metabolizers. Both of these mutations introduce stop
5 codons resulting in a truncated and non-functional enzyme.

CYP2C19 metabolizes a variety of compounds including the tricyclic antidepressants amitriptyline, imipramine and clomipramine, the sedatives diazepam and hexobarbital, the gastric proton pump inhibitors,
10 omeprazole, pantoprazole, and lansoprazole, as well as the antiviral nelfinavir mesylate, the antimalarial drug proguanil and the β -blocker propranolol.

Induction and Inhibition

CYP2C19 is inhibited by fluconazole, fluvoxamine,
15 fluoxetine, sertraline, and ritonavir. It is induced by rifampin.

Inter Ethnic Differences

The occurrence of the poor metabolizer phenotype for CYP2C19 shows a large inter ethnic variability. Poor
20 metabolizers make up less than 4% of the European and white American populations. While the Korean population has a poor metabolizer frequency of 12.6%, the Chinese 17.4% and the Japanese 22.5%. In addition, the CYP2C19 mutant alleles demonstrate interethnic variability with
25 CYP2C19*2 frequency ranging from 28.9% in the Chinese population to only 13% in European-American population. The CYP2C19*3 allele is absent from the European-American or African-American populations, while occurring at a

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frequency of 11.7% in both the Korean and Japanese populations.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Omeprazole

As an example, the benefit of CYP2C19 metabolic phenotyping in drug dosing is evident in the case of omeprazole. Omeprazole is a drug used in the treatment of *Helicobacter pylori* (*H pylori*) infections in conjunction with amoxicillin, and is cleared from the body via a CYP2C19 metabolic pathway. Studies have observed higher eradication rates of in CYP2C19 poor metabolizers. Therefore, extensive metabolizers may require higher doses of omeprazole to achieve the same level of *H pylori* eradication observed in poor metabolizers. For these reasons, the utility of a reliable phenotyping test for CYP2C19 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective treatment regimes for individuals on an individual basis.

Direct Phenotypic Determinants of CYP2C19

In accordance with an embodiment of the present invention, the ratio of S-(+)mephenytoin and R-(-)mephenytoin in an urine sample may be used to provide a determination of an individual's CYP2C19 phenotype. These metabolites are used as quantitative markers in the

determination of a CYP2C19 phenotype on the basis of the use of the preferred probe substrate mephenytoin. However, it is fully contemplated that the present invention is not limited in any respect thereto. The structure of R-(-) and S-(+) mephenytoin and 4-hydroxymephenytoin are illustrated in Fig. 5.

The chiral ratio of S-(+)mephenytoin and R-(-)mephenytoin metabolites, used to determine the CYP2C19 phenotype of the individual, is as follows:

$$\frac{\text{S-(+)Mephenytoin}}{\text{R-(-)Mephenytoin}}$$

Chiral ratios of close to unity (>0.8) are indicative of fast CYP2C19 metabolizers.

Indirect Phenotypic Determinants of CYP2C19 (Genotyping)

As mentioned previously the CYP2C19 has two predominant variant alleles, which account for all Japanese poor metabolizers and 83% of Caucasian poor metabolizers. Studies have demonstrated an excellent correlation between a homozygous presence of mutant alleles and poor metabolizer status. An example of a procedure for genotyping CYP2C19 involves a series of polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2C19*1 allele (Furuta et al. (1999) Clin Pharmacol Thera 65(5):552-561; Tanigawara et al. (1999) Clin Pharmacol Thera 66(5):528-5534). PCR amplification of exon 5 or exon 4 for CYP2C19*2 and CYP2C19*3 respectively are performed using the following primers:

CYP2C19*2 Exon 5 Primers

5'-AATTACAACCAGAGCTTGGC-3'

5'-TATCACTTTCCATAAAAGCAAG-3'

5 CYP2C19*3 Exon 4 Primers

5'-AACATCAGGATTGTAAGCAC-3'

5'-TCAGGGCTTGGTCAATATAG-3'

The presence of the G681A mutation in CYP2C19*2 is then detected by digestion with the *Sma*I restriction enzyme. The wild type allele will produce a 120 and a 49 bp fragment, while the CYP2C19*2 allele will remain uncleaved. The CYP2C19*3 allele is detected by incubating the exon 4 PCR product with *Bam*HI. The wild type allele will produce a 233 bp and a 96 bp fragment while the CYP2C19*3 allele will remain uncleaved.

Extensive metabolizing phenotype is assigned to those individuals with at least one allele encoding a functional enzyme. The poor metabolizing phenotype is assigned to individuals lacking two or more functional CYP2C19 alleles.

CYP 2C9

The CYP2C9 family of metabolic enzymes accounts for approximately 8% of the metabolic enzymes in the liver. CYP 2C9 has been postulated as participating in approximately 15% of drug metabolism.

Polymorphism

Individuals are genetically polymorphic with respect to CYP 2C9 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers. 5 Three genetic polymorphisms have been definitively identified, one wild type (CYP2C9*1) and two mutant (CYP2C9*2 and CYP2C9*3). The CYP2C9*2 allele was found to result in 5-10 fold increase in expression of mRNA and have 3-fold higher enzyme activity for metabolism of 10 phenytoin and tolbutamide. Conversely, this genotype appears to have a lower level of activity for the metabolism of S-warfarin. The CYP2C9*3 allele appears to demonstrate decreased metabolic activity against all three of these substrates.

15 CYP2C9 metabolizes a variety of compounds including S-warfarin, phenytoin, tolbutamide, tienilic acid, and a number of nonsteroidal antiinflammatory drugs such as diclofenac, piroxicam, tenoxicam, ibuprofen, and acetylsalicylic acid.

20 Induction and Inhibition

CYP2C9 is inhibited by fluconazole, metronidazole, miconazole, ketoconazole, itaconazole, ritonavir, clopidrogel, amiodarone, fluvoxamine, sulfamthoxazole, fluvastatin and fluoxetine. It is induced by rifampin and 25 rifabutin.

Inter Ethnic Differences

The CYP2C9 genotypes demonstrate marked inter ethnic variability. The CYP2C9*2 is absent from Chinese and Taiwanese populations and present in only 1% of

African American populations, but accounts for 19.2% of the British population and 8% of Caucasians. CYP2C9*3 is rarer and is present in 6% of Caucasian, 2% of Chinese, 2.6% of Taiwanese and 0.5% of African-American populations.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

10 **S-warfarin**

As an example, the benefit of CYP2C9 metabolic phenotyping in drug dosing is evident in the case of S-warfarin. S-warfarin is an anticoagulant drug. Studies have demonstrated that the presence of either CYP2C*2 or CYP2C9*3 haplotypes results in a decrease in the dose necessary to acquire target anticoagulation intensity. In addition, these individuals also suffered from an increased incidence of bleeding complications. Therefore, the CYP2C9 gene variants modulate the anticoagulant effect of the dose of warfarin prescribed. For these reasons, the utility of a reliable test for CYP2C9 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective treatment regimes for individuals on an individual basis.

Direct Phenotypic Determinants of CYP2C9

In accordance with an embodiment of the present invention, the ratio of (S)-ibuprofen and its carboxylated metabolite, (S)-2-carboxyibuprofen in a urine sample may

be used to provide a determination of an individual's CYP2C9 phenotype. These metabolites are used as quantitative markers in the determination of a CYP2C9 phenotype on the basis of the use of the preferred probe substrate (S)-ibuprofen. The structures of (S)-ibuprofen and its metabolite (S)-2-carboxyibuprofen are illustrated in Fig. 6. However, it is fully contemplated that the present invention is not limited in any respect thereto. In fact, due to the nature of the substrate specific alterations caused by the individual CYP2C9 mutations, multiple probe substrates may be necessary for a completely informative phenotypic determination of CYP2C9.

The molar ratio of (S)-ibuprofen and its (S)-2-carboxyibuprofen metabolite, used to determine the CYP2C9 phenotype of the individual, is as follows:

$$\frac{\text{(S)-ibuprofen}}{\text{(S)-2-carboxyibuprofen}}$$

Indirect Phenotypic Determinants of CYP2C9 (Genotyping)

As mentioned previously the CYP2C9 has two predominant variant alleles, CYP2C9*2 and CYP2C9*3. An example of a procedure for genotyping CYP2C9 involves a series of polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2C9*1 allele (Taube et al. (2000) Blood 96(5):1816-1819). PCR amplification of exon 3 for CYP2C9*2 is performed using the following primers:

CYP2C9*2 Exon 3 Primers

5'-CAATGGAAAGAAATGGAAGGAGGT-3'

5'-AGAAAGTAATACTCAGACCAATCG-3'

5 A forced mismatch was included in the penultimate
base of the forward primer to create a restriction site
for the *Ava*II digestion. The PCR product from this
amplification is 251 bp in length. After *Ava*II digestion
the CYP2C9*1 (wt) allele produces 170 and 60 bp fragments.
10 The CYP2C*2 allele produces a 229 bp fragment.

 The CYP2C9*3 allele does not naturally destroy or
produce a restriction site. Therefore, a restriction site
was introduced into the forward primer such that the
adenosine at position 1061 (A1061) in combination with the
15 mismatch creates a restriction site for the *Nsi*I
restriction enzyme. Therefore the PCR amplified fragment
of the CYP2C9*1 (wt) allele would have a restriction site
at A1061. Conversely, the mutation of A1061C in CYP2C9*3
removes this restriction site. The forward primer also
20 includes a natural *Ava*II restriction sequence. The reverse
primer also has a forced mismatch at 1186 to provide a
restriction site for the *Nsi*I restriction enzyme (PCR
amplified fragments from both the CYP2C9*1 and CYP2C9*3
alleles will have this restriction site). The PCR product
25 for this set of primers prior to restriction enzyme digest
is 160 bp in length. Following restriction digest with
*Nsi*I and *Ava*II, the CYP2C9*1 allele produces a 130 bp
fragment and the CYP2C9*3 allele produces a 140 bp
fragment.

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CYP2C9*3 Primers

5'-TGCACGAGGTCCAGAGATGC-3'

5'-AGCTTCAGGGTTTACGTATCATAGTAA-3'

5

Due to the substrate specific alterations in enzyme activity resulting from the two allelic variants, the phenotypic determination will be correlated on an individual substrate basis.

10

CYP2D6

CYP2D6 constitutes 1-3% of the total CYP 450 enzymes in the human liver. CYP2D6 has been postulated as participating in ~20% of drug metabolism.

15 **Polymorphism**

CYP2D6 was the first P450 enzyme to demonstrate polymorphic expression in humans. Three metabolic phenotypes can be distinguished: poor, (PM), extensive (EM) and ultraextensive (UEM) phenotypes. The CYP2D6 gene is extensively polymorphic. For example, a 1997 study documented 48 mutations and 53 alleles of the CYP2D6 gene in a screen of 672 unrelated individuals. Examples of alleles with normal (extensive), wild-type function are CYP2D6*1, CYP2D6*2A, and CYP2D6*2B; alleles resulting in an absence of function are CYP2D6*3, CYP2D6*4A, CYP2D6*4B, CYP2D6*5, CYP2D6*6A, CYP2D6*6B, CYP2D6*7, CYP2D6*8, CYP2D6*11 and CYP2D6*12; and alleles resulting in a reduced function are CYP2D6*9, CYP2D6*10A, and CYP2D6*10B. The ultraextensive phenotype appears to arise from the

presence of multiple copies of the CYP2D6 gene (for example, one individual was identified with 13 copies of the gene).

CYP2D6 metabolizes a large variety of drugs and dietary constituents including, but not limited to the following:

Antiviral agents:

Efavirenz, nevirapine, ritonavir, saquinovir, nelfinavir mesylate, and indinavir

10 Psychotropic drugs:

amiflamine, amitriptyline, clomipramine, clozapine, desipramine, haloperidol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphenazine, remoxipride, thioridazine, tomoxetine, 15 trifluperidol, zuclopenthixol, risperidone, and fluoxetine.

Cardiovascular agents:

aprimidine, bufuralol, debrisoquine, encainide, flecainide, guanozan, indoramin, metoprolol, mexiletin, n- 20 propylamaline, propafenone, propranolol, sparteine, timolol, and verapamil.

Miscellaneous agents:

chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, and phenformin.

25 In addition, CYP2D6 is involved in the metabolism of many carcinogens, however, as yet it is not reported as the major metabolizer for any. In one study it has been shown that individuals who are fast CYP2D6 metabolizers

and slow N-acetylators are at a greater risk for hepatocellular cancer (OR=2.6; 95% CI=1.6-4).

Induction and Inhibition

CYP2D6 is inhibited *in vitro* by quinidine and by
5 viral protease inhibitors as well as by appetite suppressant drugs such as D- and L-fenfluramine.

Inter Ethnic Differences

The activity of CYP2D6 varies broadly in a given
10 population. Poor (PM), extensive (EM) and ultraextensive (UEM) phenotypes of CYP2D6 have been distinguished. The CYP2D6 gene is inherited as an autosomal recessive trait and separates 90 and 10% of the white European and North American population into extensive (EM) and poor (PM)
15 metabolizer phenotypes, respectively. In another study the percentage of PM in different ethnic populations was observed, and white North Americans and Europeans were found to have 5-10% PM's, African-American, 1.8%, Native Thais, 1.2%, Chinese 1%, and Native Malay populations,
20 2.1%, while the PM phenotype appears to be completely absent in the Japanese population. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic
25 population to another.

Dextromethorphan/Antidepressants

An example of the need for phenotyping in drug dosing is the case of dextromethorphan. Dextromethorphan is a nonopioid antitussive with psychotropic effects. However, dextromethorphan doses range from 0 to 6 mg/kg based on individual individual tolerance. Dextromethorphan is activated via the CYP2D6 metabolizing system. Dextromethorphan produced qualitatively and quantitatively different objective and individual effects in poor vs. extensive metabolizers (mean performance +/-SE, 95+/-0.5% for EMs vs. 86+/-6% for PMs; p<0.05). Another important class of drugs for CYP2D6 phenotyping is the tricyclic antidepressants. Both the PM and UEM phenotypes of CYP2D6 are at risk of adverse reactions. PM individuals given standard doses of these drugs will develop toxic plasma concentrations, potentially leading to unpleasant side effects including dry mouth, hypotension, sedation, tremor, or in some cases life-threatening cardiotoxicity. Conversely, administration of these drugs to UEM individuals may result in therapeutic failure because plasma concentrations of active drugs at standard doses are far too low. For, these reasons, the utility of a reliable phenotyping test for CYP2D6 is evident

Phenotypic Determinants of CYP2D6

Different probe substrates can be used to determine the CYP2D6 phenotype (dextromethorphan, debrisoquine, bufuralol, antipyrine, theophylline and hexobarbital). In accordance with the present invention,

suitable probe substrates include without limitation, dextromethorphan, debrisoquine, and bufuralol.

Of these dextromethorphan is the preferred probe substrate. The structure of dextromethorphan and its demethylated metabolite dextrorphan are illustrated in Fig. 7.

In accordance with the present invention, the molar ratio of dextromethorphan and its metabolite is used to determine the CYP2D6 phenotype of the individual as follows:

$$\frac{\text{dextromethorphan}}{\text{dextrorphan}}$$

An antinode of 0.30 is used to differentiate between extensive and poor metabolizers whereby an antinode of less than 0.30 indicates an extensive metabolizer and greater than 0.30 indicates a poor metabolizer.

Indirect Phenotypic Determinants of CYP2D6 (Genotyping)

As mentioned previously the CYP2D6 gene is extensively polymorphic with one study identifying 48 mutations and 53 alleles. An example of a procedure for genotyping CYP2D6 involves the amplification of the entire CYP2D6 coding region (5.1kb product) by XL-PCR using specific primers. This product is then used for a series of polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2D6*1 allele (Garcia-Barceló et al. (2000) Clinical Chemistry 46(1):18-23). For example, to detect

the C188T transition mutation the following primers can be used to first amplify the CYP2D6 gene and then the specific region of the mutation:

5 Full CYP2D6 gene

5'-CCAGAAGGCTTTGCAGGCTTCA-3'

5'-ACTGAGCCCTGGGAGGTAGGTA-3'

C188T Mutation

5'-CCATTTGGTAGTGAGGCAGGTAT-3'

10 5'-CACCATCCATGTTTGCTTCTGGT-3'

The presence of the C188T mutation is then detected by digestion with the *HphI* restriction enzyme.

In general, the most frequent mutations are examined and these correspond to the most frequent alleles and genotypes.

Extensive metabolizing phenotype is assigned to those individuals with at least one allele encoding a functional enzyme. The poor metabolizing phenotype is assigned to individuals lacking two or more functional CYP2D6 alleles.

CYP2E1

CYP2E1 constitutes approximately 5% of the total CYP 450 enzymes in the human liver.

Polymorphism

25 The CYP2E1 gene has been demonstrated to be polymorphic in the human population. Studies have demonstrated the presence of 10 CYP2E1 alleles (one wt

CYP2E1*1, and 9 mutant, CYP2E1*2, CYP2E1*3, CYP2E1*4, CYP2E1*5A, CYP2E1*5B, CYP2E1*6, CYP2E1*7A, CYP2E1*7B, and CYP2E1*7C). The exact relationship of these polymorphisms to CYP2E1 enzyme activity has not been clarified, however, some studies suggest that the mutant alleles CYP2E1*5A and CYP2E1*5B, result in increased transcription and increased enzyme activity.

CYP2E1 metabolizes several drugs and dietary constituents including isoflurane, halothane, methoxyflurane, enflurane, propofol, thiamylal, sevoflurane, ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, and p-nitrophenol.

In addition, CYP2E1 activates environmental pro-carcinogens especially nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, and 3-hydroxypyridine (tobacco smoke product). In one study it has been shown that individuals who have high CYP2E1 (CYP2E1*5A or CYP2E1*5B) activity are at a greater risk for gastric cancer (OR=23.6-25.7).

20 Induction and Inhibition

CYP2E1 is induced by a number of drugs and environmental factors such as cigarette smoke as well as by starvation, chronic alcohol consumption and in uncontrolled diabetes. CYP2E1 is inhibited by chlormethiazole, trans-1,2-dichloroethylene, disulferan (cimetidine) and by the isoflavonoids genistein and equol.

Induction or inhibition by environmental factors can severely alter an individual's capacity to metabolize

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certain drugs. Therefore, the present invention may find further application in the individualization of therapy whereby environmental factors are determined to effect an individual's metabolism specific to an enzyme and/or
5 metabolic pathway of interest with respect to a given drug, such as CYP2E1, for example. Furthermore, as environmental factors vary on an individual basis and over time, the present invention may be employed to detect changes in an individual's metabolism specific to an
10 enzyme and/or metabolic pathway of interest due to environmental factors at any given time, and provide valuable phenotype-specific information in the determination of a safe and efficacious individualized treatment regime. By employing the present invention on a
15 routine basis, an individual's treatment regime may be modified to account for environmental influences and maximize the effectiveness of treatment.

Inter Ethnic Differences

The proportion of CYP2E1 phenotypes varied between
20 ethnic groups and countries: the frequency of the rare c2 (CYP2E1*5A or CYP2E1*5B) allele is about 4% in Caucasians and 20% in the Japanese and a study of a separate polymorphism described a rare C allele (CYP2E1*5A or CYP2E1*6) that has a frequency of about 10% in Caucasian
25 and 25% in Japanese populations. In one study it was shown that Japanese males had much lower levels of CYP2E1 activity as compared to Caucasian males. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of

polymorphism and its antimode should not be extrapolated from one ethnic population to another.

Acetaminophen

An example of the need for phenotyping in drug dosing is the case of acetaminophen. Acetaminophen is a widely used painkiller. However, acetaminophen causes hepatotoxicity at low frequency. The hepatotoxicity is due to its transformation via CYP2E1, to a reactive metabolite (N-acetyl-p-benzoquinoneimine) which is capable of binding to nucleophiles. For these reasons, the utility of a reliable phenotyping test for CYP2E1 is evident.

Direct Phenotypic Determinants of CYP2E1

In accordance with the present invention a suitable probe substrate is, without limitation, chlorzoxazone.

In accordance with the present invention, the molar ratio of chlorzoxazone and its metabolite is used to determine the CYP2E1 phenotype of the individual as follows:

$$\frac{\text{6-hydroxychlorzoxazone}}{\text{chlorzoxazone}}$$

The structures of chlorzoxazone and its metabolite 6-hydroxychlorzoxazone are illustrated in Fig. 8.

Indirect Phenotypic Determinants of CYP2E1 (Genotyping)

As mentioned previously the CYP2E1 gene has multiple polymorphisms. An example of a procedure for genotyping CYP2E1 for the most common mutations, those

termed the *Pst*/*Rsa*I and *Dra*I mutations (allows genotyping of CYP2E1*5A, CYP2E1*5B and CYP2E1*6), involves the amplification of a fragment containing either the *Pst*I and *Rsa*I restriction sites or the *Dra*I restriction site using specific primers (Nedelcheva et al. (1996) Methods in Enzymology 272:218-225). The amplified product is then incubated with the appropriate restriction enzyme (*Pst*I or *Rsa*I/*Dra*I) and the digestion products separated electrophoretically. From an allele with wt sequence at the *Pst*I or *Rsa*I site, the 510 bp fragment produced by PCR is cleaved to a 360 bp and a 150 bp fragment. From the mutant allele the 510 bp fragment remains uncleaved. From an allele with the wt sequence at the *Dra*I mutation site, the 370 bp PCR amplified fragment is cleaved to a 240 bp and 130 bp pair of fragments, while the mutant allele is uncleaved.

*Pst*I/*Rsa*I Primers

5'-CCCGTGAGCCAGTCGAGT-3'

5'-ATACAGACCCTCTTCCAC-3'

*Dra*I Primers

5'-AGTCGACATGTGATGGATCCA-3'

5'-GACAGGGTTTCA-TCATGTTGG-3'

The CYP2E1*5A mutant allele contains both the *Rsa*I and the *Dra*I mutations, while the CYP2E1*5B mutant allele contains the *Rsa*I mutation alone. The *Rsa*I mutation has been associated with an increased expression and increased enzyme activity. Therefore, an individual with two copies

of either CYP2E1*5 allele could be considered assigned an extensive metabolizing phenotype. Conversely, the CYP2E1*2 mutation has been associated with decreased protein expression and decreased enzyme activity.

5 Therefore, a person homozygous for the CYP2E1*2 allele could be assigned a poor metabolizing phenotype.

Characterization of Multiple Phenotypic Determinants

On the basis of such enzyme-specific metabolic pathways, as exemplified hereinabove, several approaches
10 to identifying phenotypic determinants thereof have been developed in accordance with the present invention. The characterization of multiple phenotypes offers multiple applications. The determination of an individual's metabolic phenotype for a multitude of phase I (e.g.
15 cytochrome P450) and phase II (e.g. N-acetyltransferase) metabolic enzymes allows the use of this single profile for multiple applications. If a drug is metabolized by more than one enzyme, the phenotypic status of each of the enzymes may be important for first, determining if the
20 individual can safely ingest a given drug and second, determining the optimal dose for this individual if they are able to take the drug.

In the case of amonafide, it is suggested that CYP1A2 may, in addition to NAT2, play a minor but
25 nonetheless significant role in the metabolism of this drug. Accordingly, it is contemplated that the ability to characterize multiple phenotypic determinants may also play an important role in the individualization of therapy with amonafide on the basis of phenotyping.

In addition, the knowledge of multiple phenotypes will facilitate the comparison of multiple drugs within the same class or genus, where different metabolic enzymes are involved in the metabolism of these drugs. For example, consider an individual requiring a certain class of drug, of which there are three that are primarily prescribed. If one is metabolized by CYP1A2, one by CYP2D6 and the remaining drug by CYP3A4, and all individuals that are poor metabolizers of these drugs are at risk for toxicity, then the drug chosen for treating that individual may be determined on the basis of a phenotypic profile of that individual. If for example the individual is a poor metabolizer for CYP2D6 and CYP3A4, then the first drug metabolized by CYP1A2 may be the first drug to consider for treating the individual.

Another advantage to the determination of an individual's metabolic profile for multiple phenotypic determinants is the effect of a drug on the metabolic status of enzymes not primarily involved in its metabolism. For example, a drug may be metabolized by CYP2C9 and inhibit the activity of CYP3A4. If an individual has very low levels of CYP3A4 to begin with then this inhibition may have little effect on that individual's CYP3A4 phenotype. However, if the individual is an extensive CYP3A4 metabolizer this drug may profoundly alter the CYP3A4 metabolic status. This can cause enormous problems in the case of polypharmacy, where an individual may be taking multiple drugs, and the addition of one drug may affect the safety and efficacy of the pre-existing drug treatment(s).

Examples I-III hereinbelow exemplify selected protocols for characterizing these phenotypic determinants. In addition, Examples IV-VIII outline respective applications and uses for metabolic phenotyping information obtained from these protocols.

The metabolic phenotype can be determined directly (by measuring enzyme activity) or indirectly (by inferring levels of enzyme activity). In general, for direct phenotyping, a probe substrate or substrates, such as those exemplified in Table 4 are administered to an individual to be phenotyped. A biological sample, such as a urine sample is subsequently collected from the individual approximately 4 hours after administering the probe substrate(s). The urine sample is analyzed according to a ligand binding technology.

Ligand-Binding Assays

The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of both the analytical immunoassay in solution and the immunosensor on solid-state interfaces. The underlying fundamental concept of these analytical methods as ligand-binding assays is based on the observation of the products of the ligand-binding reaction between the target analyte and a highly specific binding reagent.

The development of immunoassay technology is a success story especially for the clinical laboratory and still continues to be a vibrant area of research. Further development and automation will expand the possibilities

of immunoassay analysis in the clinical sciences. Besides this, new areas for trace analyses using immunoassay were defined in the last decade: the environmental analysis of trace substances and quality control in the food industry.

5 Since these applications also need a continuous monitoring mode, the idea of an immunosensor as a continuously working heterogeneous immunoassay system, covering these features, was conceived. The immunosensor is now considered as a major development in the immunochemical

10 field. Despite an overwhelming number of papers in this field, there are only a few commercial applications of immunosensors in clinical diagnostics. The reasons are, in part, unresolved fundamental questions relating to immobilization, orientation, and specific properties of

15 the antibodies or antibody-related reagents on the transducer surface. In addition, a key issue is which clinical applications may benefit most from immunosensor devices in the routine medical laboratory. Only if there is consensus on the clinical utility of this new technique

20 can the gap between the high expectations of the developer and reality be closed. Designers of immunosensor devices must be aware of the general and special needs of laboratory medicine from new analytical techniques.

A new analyzer should be simple and "rugged" for

25 the measurement of analytes. Measurements have to be performed precisely and accurately, even under emergency conditions. The analyzer must be fully automated and capable of performing rapid measurements with turnaround times of < 1 h. Additionally, the determination of an

30 analyte should preferably be without sample pretreatment

in matrices, such as serum, plasma, urine or cerebrospinal fluid. All parameters determined with a new analyzer must meet the following criteria, which are defined in various guidelines: low imprecision, small lot-to-lot variations,
5 high analytical sensitivity, optimum analytical specificity and accuracy with long calibration stability and low interferences by drugs or normal and pathological sample components.

In the clinical laboratory, a future substitution
10 of immunoassays by immunosensors simply depends on the superiority and versatility of the new methodology. The applicability for point-of-care testing or when they are temporarily implanted into the individual additionally depends on the reliable and accurate analysis of the
15 desired analyte, without drift problems or matrix interferences. Due to the tremendously growing variety of developments, this review is not intended to be comprehensive. Hence, the main focus will be the description and assessment of reported clinical
20 applications of immunosensors. For a more thorough understanding, we refer to several excellent reviews in the last 5 years on technical aspects and the application of immunosensors in various fields. Other related reviews deal with antibody engineering developments and latest
25 immunoassay technologies.

Antibodies as bioaffinity interface for both immunoassays and immunosensors

It should first be clarified that the specificity for the measurement of analytes in all immunosensor

systems, as in the case of immunoassays, is dependent on the application of affinity complexation agents (binding molecules). This pivotal feature is shared by both technologies. New developments in protein engineering for immunoglobulins (including antibody fragments, and chimeric antibodies) or in substituting antibodies by alternative binding components (aptamers are one example) or structures (molecular imprinting is one example) will, therefore, be applicable to either technology, if available. In particular, the possibilities in antibody engineering will enable changes in the affinity and fine specificity of antibodies, as well as the expression of fragments as fusion proteins coupled to reporter molecules.

15 **Immobilization procedures for antibodies**

Antibodies have to be properly immobilized on the immunosensor surface, which is mostly part of a flow-through cell. The optimum density and adjusted (but not random) orientation of the antibodies are of paramount importance. Due to the different types of sensing surfaces, this manipulation can have benefits e.g., improvement of the reaction kinetic parameters, but also unfavorable effects (e.g., increased nonspecific binding, partly destroyed paratope). There are four different types of oriented coupling of antibodies: binding to Fc receptors such as protein A or G or recombinant ArG fusion protein on the surface; binding of other binding partners to structures, covalently linked to the Fc part of the antibody, e.g., the biotin residue on the Fc binds to surface-coated streptavidin; coupling to the solid support

via an oxidized carbohydrate moiety on the C2 Fc domain; and the binding of Fab or scFv fragments to the surface of the device via a sulfhydryl group in its C-terminal region.

5 Numerous chemical reactions can be applied to the immobilization onto solid surfaces. Defined linkages between the antibody or its carbohydrate moieties and the solid phase material (silica, silanized silica, Ta- or Ti-oxides, plastics, sepharose, and metal films) are being
10 built by glutaraldehyde, carbodiimide, succinimide ester, maleinimide, periodate or galactose oxidase. Moreover, photo-immobilization of antibodies using albumin derivatized with aryl diazirines as photolinker, is applicable. Physiosorption is not recommended due to the
15 local instability of the layer caused by the mechanical stress in the flow-through cell. An exciting new method for antibody immobilization on a quartz surface of a piezoelectric sensor is based on the deposition of an ethylenediamine plasma polymerization film on the quartz
20 crystal. This film is extremely thin and homogeneous, incorporating amino functions which may be further derivatized and linked to immunoglobulins, resulting in an orientation-controlled and highly reusable sensing surface. Another recent development is the planar-
25 supported phospho-lipid bilayer (SLB), which can be formed on solid supports by vesicle fusion and Langmuir-Blodgett methods. SLBs maintain two-dimensional fluidity and accommodate multivalent binding between surface-bound ligands and receptor molecules in solution.

For noble metal surfaces, such as gold, in particular, in optical immunosensors, self-assembling monolayer (SAM) techniques seem to be first choice. In general, a SAM is built of long-chained (C_{12} and higher) *n*-alkylthiols with derivatized organic functional groups, which are easily linked to the gold film via the thiol groups by a mechanism still not fully understood. The functional groups of the SAM crosslink with the Fc portion of the antibody (one way is via the biotin streptavidin system), whereas the self-organization of the matrix prevents the surface being individualized to nonspecific binding effects. In addition, the covalent coupling of IgG to a short-chain (thioctic or mercaptopropionic acid are two examples) SAM-modified metal surface has been shown to be an effective affinity-based layer for optical immunosensors.

Regeneration of antibody-coated sensor surfaces

Conventional homogeneous and heterogeneous immunoassays, respectively, work discontinuously. It is highly desirable, however, that immunosensor devices, applied in clinical diagnostics, are capable of quasi-continuous recording. The repeated use of disposable sensing elements may mimic a pseudocontinuous action, but this is not considered here. In true immunosensors, the analyte/antigen interaction on the sensor-coated surface is reversible. With the given short incubation times in the flow-through device, the reaction between antigen and antibody is far off the equilibrium state. Fast reversibility and high sensitivity are mutually exclusive of each other. Consistently, an adequate analytical

sensitivity is only warranted if antibodies with increased affinity $>10^{10} \text{ M}^{-1}$ or at least with highly improved on-rate are applied.

The regeneration of the binding sites of the
5 antibodies bound to the immunosensor surface needs stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the
10 immobilized antibodies and insidious drift problems.

Besides this, it must be considered that with the short reaction times between the antibodies and soluble analytes in the flow-through system, the cross-reactivities of the antibody applied can be increased. A
15 highly specific recognition of the antigen is a kinetic-controlled process due to the complexity of the conformational changes in the Fab portion of the antibody upon binding of the antigen.

There are different approaches to solve the
20 "antibody regeneration" problem: one approach is to displace the antigenic analyte by a highly concentrated solution of a related antigen with weak affinity to the surface-bound antibody. However, this depends on the availability of a suitable antigenic surrogate. This is
25 not always feasible and is only applicable to small analytes. A second approach is to use the techniques of antibody engineering to improve the chemical stability of antibodies as whole molecules or as Fab fragments. The phage display technique is such a powerful tool. This can

be helpful in the selection of antibody fragments with improved stability. Libraries of mutants of single-chain Fv fragments (scFv), comprising the variable regions of the L and H chains, joined by a peptide linker are
5 generated by a combination of site-directed and random mutagenesis. The selection can be carried out under different physical or chemical pressures to produce thermodynamically more stable scFv mutants. An interesting third approach is a pseudo-regenerating procedure for
10 immunosensors. An amperometric sensor is coated with a conducting immunocomposite, formed by a mixture of specific antibody with methacrylate monomer and graphite. After polymerization, the device is ready for use. Repeated measurements became possible if the polymer is
15 polished thoroughly with abrasive paper. These notes do not apply to immunosensors with a competitive configuration, in which antigenic compounds and not antibodies are surface-immobilized.

20 **Alternative analyte-binding compounds for immunosensor applications**

Aptamers

Aptamers are single-stranded DNA or RNA
25 oligonucleotide sequences with the capacity to recognize various target molecules with high affinity and specificity. These ligand-binding oligonucleotides mimic properties of antibodies in a variety of diagnostic formats. They are folded into unique overall shapes to

form intricate binding furrows for the target structure. Aptamers are identified by an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers may have advantages over
5 antibodies in the ease of depositing them on sensing surfaces. Moreover, due to the highly reproducible synthetic approach in any quantities, albeit the affinity constants are consistently lower than those of antibodies and the stability of these compounds is still
10 questionable, they may be particularly useful for diagnostic applications in complex biological matrices. The aptamer-based schemes are still in their infancy and it is expected that modified nuclease-resistant RNA and DNA aptamers will soon be available for a variety of
15 therapeutic and diagnostic formats. The potential of aptamers for use in biosensors has been outlined in the design of a fiber-optic biosensor using an anti-thrombin DNA aptamer, immobilized on the surface of silica microspheres and distributed into microwells on the distal
20 tip of the imaging fiber. With this device, the determination of thrombin at low concentration was possible. Exciting new possibilities are evolving by the introduction of signaling aptamers with ligand-dependent changes in signaling characteristics and catalytically
25 active so-called "apta-zymes" which would allow the direct transduction of molecular recognition to catalysis.

Anticalins

Lipocalins constitute a family of proteins for
30 storage or transport of hydrophobic and/or chemically

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sensitive organic compounds. The retinol-binding protein is an example in human physiology. It has been demonstrated that the bilin-binding protein, a member of the lipocalin family and originating from the butterfly *Pieris brassicae*, can be structurally reshaped in order to specifically complex potential antigens, such as digoxigenin, which was given as an example. These binding proteins share a conserved β -barrel, which is made of eight antiparallel β -strands, winding around a central core. At the wider end of the conical structure, these strands are connected in a pairwise manner by four loops that form the ligand binding site. The lipocalin scaffold can be employed for the construction of so-called "anticalins", which provide a promising alternative to recombinant antibody fragments. This is made by individualizing various amino acid residues, distributed across the four loops, to targeted random mutagenesis. It remains to be shown that this class of proteins is applicable in diagnostic assays and in immunosensors. Critical points that still need to be defined include the synthesis and stability of the anticalins, the magnitude of the affinity constants, and the versatility for being crafted against the large variety of ligands.

25 *Molecular imprinting techniques*

This is a technique that is based on the preparation of polymeric sorbents which are selectivity predetermined for a particular substance, or group of structural analogs. Functional and cross-linking monomers

of plastic materials, such as methacrylics and styrenes, are allowed to interact with a templating ligand to create low-energy interactions. Subsequently, polymerization is induced. During this process, the molecule of interest is entrapped within the polymer either by a noncovalent, self-assembling approach, or by a reversible, covalent approach. After stopping the polymerization, the template molecule is washed out. The resultant imprint of the template is maintained in the rigid polymer and possesses a steric (size, shape) and chemical (special arrangement of complementary functionality) memory for the template. The molecularly imprinted polymer (MIP) can bind the template (= analyte) with a specificity similar to that of the antigen-antibody interaction.

Besides the main applications in solid-phase extraction and chromatography, molecularly imprinted polymers have already been employed as nonbiological alternatives to antibodies in competitive binding assays. A series of applications for analytes, such as cyclosporin A, atrazine, cortisol, 17b-estradiol, theophylline, diazepam, morphine, and S-propranolol, suggests that molecular imprinting is a promising technique for immunoassays and immunosensors.

Immunoassay and immunosensor technologies

Immunoassays

Immunoassays use antibodies or antibody-related reagents for the determination of sample analytes. This

analytical tool has experienced an evolutionary history since 1959, when Berson and Yalow first described the radioimmunoassay (RIA) principle. In the RIA, a fixed and limited amount of antibody is reacted with a fixed and limited amount of radiolabeled antigen tracer and a variable concentration of the analyte. The selectivity of the ligand-binding of antibodies allows these biomolecules to be employed in analytical methods that are highly specific even in complex biological matrices, such as blood, plasma, or urine. By combining the selectivity of antibody-analyte interactions with the vast array of antibodies preformed in immunization processes of host animals and the availability of numerous readily detectable labels radioisotopes, enzymatically or electrochemically induced adsorbance or fluorescence or chemi-luminescence, immunoassays can be designed for a wide variety of analytes while with extraordinarily low detection limits.

Biosensors and immunosensors

A biosensor is an analytical device that integrates a biological element on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. The biological element is a layer of molecules qualified for biorecognition, such as enzymes, receptors, peptides, single-stranded DNA, or even living cells. If antibodies or antibody fragments are applied as a biological element the device is called an immunosensor. Compared to conventional analytical instruments, biosensors are characterized by an integrated

structure of these two components. Many devices are connected with a flow-through cell, enabling a flow-injection analysis (FIA) mode of operation. Biosensors combine high analytical specificity with the processing
5 power of modern electronics to achieve highly sensitive detection systems.

There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses mainly enzymes as the
10 biological compound, catalyzing a signaling biochemical reaction. The bioaffinity-based biosensor, designed to monitor the binding event itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody-related substances for
15 biomolecular recognition. In the latter two cases, these biosensors are called immunosensors.

Biosensors are extensively used as diagnostic tools, predominately in point-of-care testing. Probably the most successful commercialization of biosensors today
20 is the *in vitro* near individual measurement of capillary glucose using various hand-held systems with disposable reagent cartridges.

Immunosensor principles

25 The general immunosensor design is depicted in Fig. 10. There are four types of immunosensor detection devices: electrochemical (potentiometric, amperometric or conductometric/capacitative), optical, microgravimetric, and thermometric. All types can either be run as direct

nonlabeled or as indirect labeled immunosensors. The direct sensors are able to detect the physical changes during the immune complex formation, whereas the indirect sensors use signal-generating labels which allow more sensitive and versatile detection modes when incorporated into the complex.

There is a great variety of different labels which have been applied in indirect immunosensors. In principle they are the same labels as used in immunoassays. Among the most valuable labels are enzymes such as peroxidase, glucose oxidase, alkaline phosphatase (AP), catalase or luciferase, electroactive compounds such as ferrocene or In^{2+} salts, and a series of fluorescent labels (including rhodamine, fluorescein, Cy5, ruthenium diimine complexes, and phosphorescent porphyrin dyes). In particular, laser-induced fluorometric resonance energy transfer between two fluorophores offers methodological advantages and can be extended to fiberoptic sensing.

Although indirect immunosensors are highly sensitive due to the analytical characteristics of the label applied, the concept of a direct sensor device is still fascinating and represents a true alternative development to immunoassay systems. Its potential simplicity holds multiple advantages, making immunosensors progressive and future directed.

The present invention will be illustrated using the following examples, which are not to be seen as limiting in any way. Those skilled in the art will recognize, or be able to ascertain using no more than

routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

5

Electrochemical sensors

Potentiometric immunosensors. The Nernst equation provides the fundamental principle of all potentiometric transducers. According to this equation, potential changes are logarithmically proportional to the specific ion activity. Potentiometric transducer electrodes, capable of measuring surface potential alterations at near-zero current flow, are being constructed by applying the following methodologies.

Transmembrane potential. This transducer principle is based on the accumulation of a potential across a sensing membrane. Ion-selective electrodes (ISE) use ion-selective membranes which generate a charge separation between the sample and the sensor surface. Analogously, antigen or antibody immobilized on the membrane binds the corresponding compound from the solution at the solid-state surface and changes the transmembrane potential.

Electrode potential. This transducer is similar to the transmembrane potential sensor. An electrode by itself, however, is the surface for the immunocomplex building, changing the electrode potential in relation to the concentration of the analyte.

Field-effect transistor (FET). The FET is a semiconductor device used for monitoring of charges at the surface of an electrode, which have been built up on its metal gate between the so-called source and drain electrodes. The surface potential varies with the analyte concentration. The integration of an ISE with FET is realized in the ion-selective field-effect transistor (ISFET). This technique can also be applied to immunosensors.

10 An advantage of potentiometric sensors is the simplicity of operation, which can be used for automation, and the small size of the solid-state FET sensors. All potentiometric methods, however, are still suffering from major problems of sensitivity, being inferior to
15 amperometric transducers and nonspecific effects of binding or signaling influences from other ions present in the sample. Especially, the signal-to-noise ratio causes analytical problems, which are difficult to circumvent. Thus, a trend away from these techniques has been observed
20 in the last few years. However, the ISFET may be seen as a candidate for ultrasensitive clinical immunosensor applications, in particular, when the novel concept of differential ISFET-based measurement of the zeta potential is used. The streaming potential is a potential difference
25 in flow direction, caused by the flow of excess ions resulting from a local distortion of the charge balance. The zeta potential, directly correlated to the streaming potential, reflects the potential changes in the diffuse outer layer at the solid-liquid interface. It efficiently

reacts to protein accumulations onto sensor surfaces and, thus, is suitable for detecting immunocomplex reactions.

Amperometric immunosensors.

5 Amperometric immunosensors are designed to measure a current flow generated by an electrochemical reaction at constant voltage. There are only few applications available for direct sensing, since most protein analytes are not intrinsically able to act as redox partners in an
10 electrochemical reaction. Therefore, electrochemically active labels directly or as products of an enzymatic reaction are needed for the electrochemical reaction of the analyte at the sensing electrode. Oxygen and H₂O₂ electrodes are the most popular. An oxygen electrode
15 consists of an electrolyte-bearing chamber with a sensing Pt cathode, polarized at 0.7 V, and an Ag/AgCl reference electrode. The chamber is gas-permeable, covered by an O₂-pervious membrane.

 Besides oxygen, generated by catalase from H₂O₂
20 there are other amperometrically detectable compounds, such as ferrocene derivatives or In²⁺ salts. A novel approach is the use of the redox polymer [PVP-Os(bipyridyl)₂Cl], which is coimmobilized with specific antibodies. Additionally, there are examples for enzymes
25 with electrochemically active products. AP, for example, catalyzes the hydrolysis of phenyl phosphate or *p*-aminophenyl phosphate (4-APP) compounds, which result in electrochemically active phenol or *p*-aminophenol. Furthermore, enzymes, such as horseradish peroxidase

(HRP), glucose oxidase, glucose-6-phosphate dehydrogenase, with subsequent amperometrical oxidation of NADH and others, have also been successfully applied as labels.

The main disadvantage for amperometric immunosensors of having an indirect sensing system, however, is compensated for by an excellent sensitivity. This is due to a linear analyte concentration range compared to a logarithmic relationship in potentiometric systems. Special attention must be directed to the system-inherent transport rate limitations for redox partners on the electrode surface.

Conductometric and capacitive immunosensors.

These immunosensor transducers measure the alteration of the electrical conductivity in a solution at constant voltage, caused by biochemical enzymatic reactions which specifically generate or consume ions. The capacitance changes are measured using an electrochemical system, in which the bioactive element is immobilized onto a pair of noble metal, mostly Au or Pt, electrodes. There are only few clinical applications available, as the high ionic strength of biological matrices makes it difficult to record the relatively small net conductivity changes caused by the signaling reaction. To circumvent this problem, recently, an ion-channel conductance immunosensor, mimicking biological sensory functions, was developed. The basis of this technique is the fact that the conductance of a population of molecular ion channels, built of tethered gramicidin A and aligned across a lipid

bilayer membrane, is changed by the antibody-antigen binding event. Different applications using various antibodies, linked to the ion-channel complex, are given.

Another approach is the measurement of changes of
5 the surface conductivity. For example, a conductometric immunosensor for the determination of methamphetamine (MA) in urine was recently developed. Anti-MA antibodies were immobilized onto the surface of a pair of platinum electrodes. The immunocomplex formation caused a decrease
10 in the conductivity between the electrodes. The measurement of the reciprocal capacitance, performed at alternating voltage, is advantageous compared to conductometric devices, and serves two purposes. The first is to test the insulating monolayer on the sensor noble metal surface.
15 Self-assembling monolayers, have insulating properties. Besides this, they prevent the immunosensor from being affected by nonspecific binding phenomena. Even minor desorption of the monolayer results in an essential increase in capacitance. Thus, the actual quality of the
20 device can be checked. The second application is the measurement of changes in the effective dielectric thickness of the insulating layer during antigen binding, when antibodies are linked to the alkylthiol layer. Of course, this is on condition that the v-substitution of
25 the alkylthiol monolayer does not compromise the insulation. Hence, a marked decrease of the electrical capacitance is observed and is used to quantitate the analyte. The destructive influence of lateral diffusion on nanostructured monolayers is prevented by use of the
30 spreader-bar technique.

Optical sensors

Optical immunosensors are most popular for bioanalysis and are today's largest group of transducers. This is due to the advantages of applying visible radiation compared to other transducer techniques. Additional benefits are the nondestructive operation mode and the rapid signal generation and reading. In particular, the introduction of fiber bundle optics ("optodes") as optical waveguides and sophisticated optoelectronics offers increased versatility of these analytical devices for clinical applications.

Changes in adsorption, fluorescence, luminescence, scatter or refractive index (RI) occur when light is reflected at sensing surfaces. These informations are the physical basis for optical sensor techniques. Usually, applied detectors are photodiodes or photomultipliers.

There are numerous applications of either direct label-free optical detection of the immunological reaction, of labeled immunospecies, or of the products of enzymatic reactions. Most labels are fluorescent, but bio- and chemiluminescence species are also possible. It is worth mentioning that the label-free evanescence wave-related sensors explicitly represent an elegant methodology, which is a valuable alternative to sophisticated immunoassays. Nevertheless, label-free systems are prone to unsolved problems, such as nonspecific binding effects and poor analytical sensitivity to analytes with low molecular weight.

Kubitschko et al. noted that despite the efforts, all immunosensors are still one magnitude less sensitive than commercial immunoassays for determining analytes in human serum, particularly those with low molecular weight. They
5 claim the use of mass labels, such as latex particles, in order to enhance the signal. The authors demonstrated the optimization of a nanoparticle enhanced bidiffractive grating coupler immunosensor for the detection of thyroid-stimulating hormone (TSH, MW 28,000 Da). The excellent
10 performance characteristics of this sensor clearly showed how future devices should work. The problem of unspecific binding, however, can also be controlled by applying a reference sensing region on the chip.

15 *Total internal reflection spectroscopy (TIRS).*

The common principle of the following analytical devices is that in an optical sensor with two materials with different refractive indices (RI), total internal reflection occurs at a certain angle of the light beam
20 being directed through the layer with the higher RI towards the sensing interface. By this, an evanescence wave is generated in the material with the lower RI. This wave, being an electrical vector of the wavelength of the incident light beam, penetrates further into the medium
25 with exponentially attenuated amplitude. Biomolecules attached in that portion of the medium will interact inevitably with the evanescent wave and, therefore, lead to a distinctive diminution of the reflected light. The resolution is directly proportional to the length of

interaction. Infrared spectrometers, measuring attenuated total reflectance, are commonly built in the Kretschmann configuration: an optically absorbing film at the sensor's surface enables the measurement of the attenuated light intensity as a function of the wavelength of the incident beam. For total internal reflection fluorescence (TIRF), analytics benefit from the fact that incident light excites molecules with fluorescence characteristics near the sensor surface creating a fluorescent evanescent wave. The emerging fluorescence is finally detected. The technique has been developed mainly for an optical detection of fluorescence-labeled antibodies or antigens. In the latter case, the fluorescence capillary fill device (FCFD) technique is worth mentioning. The FCFD is designed by using a planar optical waveguide and a glass plate separated from each other by a capillary gap. Fluorophore-labeled antigen is attached on the surface of the glass plate, whereas antibodies are immobilized on the surface of the optical waveguide.

Another phenomenon, the optical diffraction, is used by the optical biosensor assay (OBATM) system: biomolecules are attached to the surface of a silanized wafer. The protein-coated surface is illuminated through a photo mask to create distinct periodic areas of active and inactive protein. Upon illumination with laser light, the diffraction grating caused by the ligand-binding process diffracts the incident light. An analyte-free negative sample does not result in diffraction because no antigen-antibody binding occurred creating the diffraction

grating. The presence or absence of a diffraction signal differentiates between positive and negative samples. The intensity of the signal provides a quantitative measure of the analyte concentration.

5

Ellipsometry

If linearly polarized light of known orientation is reflected at oblique incidence from a surface, the reflected light is elliptically polarized. The shape and orientation of the ellipse depend on the angle of incidence, the direction of the polarization of the incident light, and the reflection properties of the surface. On adsorption of biomolecules onto a planar solid surface, phase and amplitude of the reflected light are altered and can be recorded by ellipsometric techniques. These changes in the polarization of the light are due to the alterations of the RI and the coating thickness. There are only few applications, such as the study of a cholera toxin-ganglioside GM1 receptor-ligand reaction, which were carried out using an ellipsometer.

Optical dielectric waveguides.

Optical waveguides are glass, quartz or polymer films or fibers made of high RI material embedded between or in lower index dielectric materials. If a linearly polarized helium-neon laser light wave, introduced into the high index film or fiber, arrives at the boundary at an angle which is greater than the critical angle of total

reflection, it is confined inside the waveguide. Similar to surface plasmon resonance, an evanescent field develops at the sensor's surface. In this case, however, the evanescent field is generated by the excitation of the light itself in the dielectric layer. Most of the laser light is transmitted into the device and multiple reflections occur as it travels through the medium if a bioactive substance is placed over the surface. Some of the light, however, penetrates the biolayer. This light is reflected back into the waveguide with a shift in phase interfering with the transmitted light. Thus, changes in properties of the biolayer can be followed by detecting the changes in interference.

Waveguides are often made in the form of fibers. These fiber-optic waveguide systems offer advantages for sensors when being used for hazardous analysis. Planar waveguide systems are also applicable for interferometers. They use laser light directed towards the surface of the waveguide with the attached biomolecules, which is subsequently split into two partial electrical (TE) and magnetic (TM) fieldwaves, perpendicular to each other. The interaction with the sample surface changes the relative phase between TE and TM by the different RI and surface thickness values. Various configurations, such as the Fabry-Perot monomode channel interferometer, the Mach-Zehnder interferometer or the related two-mode thin-film waveguide difference interferometer, have been successfully established.

Another technique uses thin corrugations etched into the surface of a waveguide. This grating coupler

device allows the measurement of the coupling angle of either the input or output laser beam. Both beams are correlated to the RI within the evanescent field at the sensor's surface. Recently, a long-period grating fiber immunosensor has proven to be sensitive (enabling analyses down to the nanomolar range) and reproducible. Grating couplers are also used for optical waveguide lightmode spectroscopy (OWLS). The basic principle of the OWLS method is that linearly polarized light is coupled by a diffraction grating into the waveguide layer. The incoupling is a resonance phenomenon that occurs at a defined angle of incidence that depends on the RI of the medium covering the surface of the waveguide. In the waveguide layer, light is guided by total internal reflection to the edges where it is detected by photodiodes. By varying the angle of incidence of the light, the mode spectrum is obtained from which the effective RIs are calculated for both TE and TM.

20 *Surface plasmon resonance (SPR).*

Among the different detection systems, SPR is the most popular one. There are two leading systems on the market: the BIAcore™ systems from Biacore (Uppsala, Sweden) and the IAsys™ from Fisons Applied Sensor Technology (Cambridge, UK). Other systems with small market positions are the BIOS-1 from Artificial Sensing Instruments (Switzerland), the SPR-20 from Denki Kagaku Keiki (Japan), the SPEETA from Texas Instruments (USA), the IBIS from Windsor Scientific (UK) and the DPX from Quantech (USA). The first two commercial evanescence-wave

devices are widespread in research laboratories due to the sophisticated apparatus and userfriendly control software. The BIAcore™, however, has the biggest market position.

The general principle of SPR measurement 80 is depicted in Fig. 11. Polarized light is directed from a layer of high RI towards a layer with low RI to result in total internal reflection. The sample is attached to the layer of low RI. At the interface between the two different media, a thin approximately 50 nm gold film is interposed. Although light does not propagate into the low RI medium, the interfacial intensity is not equal to zero. The physical requirement of continuity across the interface is the reason for exciting the surface electrons "plasmons" in the metal film by the light energy. As a result, the electrons start oscillating. This produces an exponentially decaying evanescent wave penetrating a defined distance into the low RI medium, which is accountable for a characteristic decrease in the intensity of the reflected light. Hence, a direct insight in changes of the RI at the surface interface is made possible by monitoring the intensity and the resonance angle of the reflected light, caused by the biospecific interactions which took place there. Whereas in the BIAcore™ system, the light affects the sensing layer only once, there are several propagation contacts in the IAsys™ due to the device's resonant mirror configuration. The BIAcore™ SPR apparatus is characterized by a sensitive measurement of changes of the RI when polarized laser light is reflected at the carboxy-methylated dextranactivated device

interface. The IAsys™ SPR device also uses a carboxy-methylated dextran-activated surface. Its dextran layer, however, is not attached to a gold surface, but to titanium, which forms a high refractive dielectric resonant layer. The glass prism is not attached tightly on the opposite side of the titanium layer, making space for an interposed silica layer of low RI. By this layer, the laser light beam couples into the resonant layer via the evanescent field. Therefore, the IAsys™ is seen as a combination of SPR resonant mirror with waveguide technology. As a result, no decrease in the reflected light intensity at resonance is observed in this system. The specific signal is the change in the phase of the reflected polarized light

Differential SPR, a novel modification of a SPR immunosensor, improves further the sensitivity of the sensor by applying a modulation of the angle of light incidence. The reflectance curve is measured with a lock-in amplifier and recorded in the first and second derivative.

Light is directed from a prism with a RI towards a layer with low RI, resulting in total internal reflection. Although light does not propagate into the medium, the interfacial intensity is not equal to zero. Physical requirements of continuity across the interface are the cause of excitation of surface plasmons in the metal film by the light energy, causing them to oscillate. This produces an exponential evanescent decaying, which penetrates a defined distance into the low-index medium

and results in a characteristic decrease in reflected light intensity.

Microgravimetric sensors

5 A direct measurement of mass changes induced by the forming of antigen/antibody complexes is also enabled by acoustic sensors. The principle of operation is based on the propagation of acoustic shear waves in the substrate of the sensor. Phase and velocity of the
10 acoustic wave are influenced by the specific adsorption of antibody molecules onto the antigen-coated sensor surface. Piezoelectric materials, such as quartz (SiO_2), zinc oxide (ZnO) or others resonate mechanically at a specific ultrasonic frequency in the order of tens of megahertz
15 when excited in an oscillating electrical field. The resonant frequency is determined by the distance between the electrodes on both sides of the quartz plate, which is equal to the thickness of the plate and the velocity of the acoustic wave within the quartz material. In other
20 words, electromagnetic energy is converted into acoustic energy, whereby piezoelectricity is associated with the electrical polarization of materials with anisotropic crystal structure. The most applied technique for monitoring the acoustic wave operation is the oscillation
25 method. This means a configuration in which the device constitutes the frequency-controlling element of a circuit. The oscillation method measures the series resonant frequency of the resonating sensor.

The microgravimetric sensor devices are divided into quartz crystal microbalance (QCM) devices applying a thickness-shear mode (TSM), and devices applying a surface acoustic wave (SAW) detection principle. These sensors
5 have reached considerable technical sophistication.

Additional bioanalytical application devices include the flexural plate wave (FPW), the shear horizontal acoustic plate (SH-APM), the surface transverse wave (STW) and the thin-rod acoustic wave (TRAW)

10 There are considerable similarities between the physical principles of QCM and SPR sensors, even when there are fundamental differences. Both QCM and SPR are wave-propagation phenomena and show resonance structure. The elastic QCM wave and the surface plasmon wave are
15 nonradiative, i.e., an evanescent wave exists. Changes of physical properties within the evanescent field lead to a shift of resonance. Thus, a linear approximation of the physical relationship is allowed for immunological application in immunosensors.

20

The TSM sensor

The TSM sensor consists of an AT-cut piezoelectric crystal disc, most commonly of quartz because of its chemical stability in biological fluids and resistance to
25 extreme temperatures. The disc is attached to two metal electrodes on opposite sides for the application of the oscillating electric field. The TSM is run in a range of 5-20 MHz. The schematic design of a typical TSM device shown in Fig. 12. Advantages are, besides the chemical

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inertness, the low cost of the devices and the reliable quality of the mass-produced quartz discs. Major drawbacks of the system are the insensitivity for analytes with a molecular weight >1000 Da, and, as seen in all label-free immunosensor systems, nonspecific binding interferences. Nonspecific binding effects are hard to distinguish from authentic binding events due to the fact that no reference line can be placed in the sensor device. For a SH-APM device, however, by appropriately selecting the device frequency, these spurious responses can be suppressed. This sensor is applicable for measurements in human serum matrix.

One of the first applications of TSM technology was an immunosensor for human immunodeficiency virus (HIV) serology. This sensor was realized by immobilizing recombinant viral peptides on the surface of the transducer and by detecting anti-HIV antibodies directly in human sera.

20 The SAW sensor.

SAW sensors use thick ST-cut quartz discs and interdigitated metal electrode arrays that generate acoustic Rayleigh waves in both directions from the interdigital electrodes, their transmission being attenuated by surface-attached biomolecules. The oscillation frequency of a SAW sensor ranges from 30 to 500 MHz. The operation of SAW immunosensors with biological samples is compromised by the fact that the surface wave is considerably attenuated in the liquid

phase. Thus, the domain of this technique is most likely restricted to gas phase operations.

The present invention is exemplified as an ELISA as described hereinbelow for corresponding probe substrate
5 and or metabolites and the molar ratios thereof calculated to reveal the individual phenotypes.

Table 4
Examples of Enzymes and Corresponding Probes Drugs

Enzyme	Probe substrate
NAT1	p-aminosalicylic acid
NAT2	Caffeine
CYP1A2	Caffeine
CYP2A6	Coumarin
CYP2C9	Diclofenac
CYP2C19	s-ibuprofen
CYP2D6	Dextromethorphan
CYP2E1	Chlorzoxazone
CYP3A4	Midazolam

10

In Example I, a detailed description of the synthesis of probe substrate and metabolite derivatives and the ELISA development for N-acetyltransferase (NAT2) and CYP1A2 are illustrated. The materials and methods, and
15 the overall general process described for the development of the NAT2 and CYP1A2 ELISA method and kit for metabolic phenotyping can be and will be applied to the development of the metabolic phenotyping ELISA kits for other metabolic enzymes including NAT1, CYP1A2, CYP2A6, CYP2D6,

CYP2E1, CYP3A4, CYP2C9 and CYP2C19, as well as a multi-determinant metabolic phenotyping system and method.

5

EXAMPLE I

10 Determination of Phenotypic Determinants by ELISA

NAT2

20 Different probe substrates can be used to determine the NAT2 phenotype (Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, **47**:470-477; Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, **49**:648-657). In accordance with the present invention caffeine is the preferred probe because it is widely consumed and relatively safe (Kalow, W. et al. (1993) *Clin. Pharmacol. Ther.*, **53**:503-514). In studies involving this probe, the phenotype has been generally determined from ratios of the caffeine metabolites 5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU) and 1-methylxanthine (1X). In these studies, the individuals are given an oral dose of a caffeine-containing substance, and the urinary concentrations of the target metabolites determined by HPLC (Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, **47**:470-477; Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, **49**:648-657) or CE (Lloyd, D. et al. (1992) *J. Chrom.*, **578**:283-291).

The number of clinical protocols requiring the determination of NAT2 phenotypes is rapidly increasing and in accordance with the present invention, an enzyme linked immunosorbent assay (ELISA) was developed for use in these
5 studies (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) *J. Pharm. Biomed. Anal.*, **13**:1079-1086). ELISAs have been successfully applied in the determination of low amounts of drugs and other antigenic components in plasma and urine samples, involve no extraction steps and are
10 simple to carry out.

In accordance with the present invention, antibodies were raised in animals against two caffeine metabolites [5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU), and 1-
15 methyl xanthine (1X)] present in urine samples of an individual collected after drinking coffee. Their ratio provides a determination of an individual's N-acetylation (NAT2) phenotype. Subsequently, there was developed a competitive antigen enzyme linked immunosorbent assay
20 (ELISA) for measuring this ratio using these antibodies.

The antibodies of the present invention can be either polyclonal antibodies or monoclonal antibodies raised against two different metabolites of caffeine, which allow the measurement of the molar ratio of these
25 metabolites.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the acetylation phenotype of the individual as follows. Individuals with a ratio less than 1.80 are slow
30 acetylators.

Materials and Methods

Materials

Cyanomethylester, isobutyl chloroformate, dimethylsulfate, sodium methoxide, 95% pure, and tributylamine were purchased from Aldrich (Milwaukee, WI, USA); horse radish peroxidase was purchased from Boehringer Mannheim (Montreal, Que., Canada); Corning easy wash polystyrene microtiter plates were bought from Canlab (Montreal, Que., Canada); o-methylisourea hydrochloride was obtained from Lancaster Laboratories (Windham, NH, USA); alkaline phosphatase conjugated to goat anti-rabbit IgGs was from Pierce Chemical Co. (Rockford, IL, USA); bovine serum albumin fraction V initial fractionation by cold alcohol precipitation (BSA), complete and incomplete Freund's adjuvants, diethanolamine, 1-methylxanthine, p-nitrophenol phosphate disodium salt, o-phenylenediamine hydrochloride; porcine skin gelatin, rabbit serum albumin (RSA); Sephadex™ G25 fine, Tween™ 20 and ligands used for testing antibodies' cross reactivities were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Whatman™ DE52 diethylaminoethyl-cellulose was obtained from Chromatographic Specialties Inc. (Brockville, Ont., Canada). Dioxane was obtained from A&C American Chemicals Ltd. (Montreal, Que., Canada) and was refluxed over calcium hydride for 4 hours and distilled before use. Other reagents used were of analytical grade.

Synthetic procedures

The synthetic route for the production of AAMU-hemisuccinic acid (VIII) and 1-methylxanthine-8-propionic acid (IX) is presented in Fig. 13.

5 **Synthesis of 2-methoxy-4-imino-6-oxo-dihydropyridine (III)**

Compound III is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, **90**:2272-2276) as follows. . To a 250 mL round bottom flask 12.2 g of o-methylisourea hydrochloride (110.6 mmol), 11.81 mL methylcyanoacetate (134 mmol), 12.45 g of sodium methoxide (230.5 mmol) and 80 mL of methanol are added. The suspension is stirred and refluxed for 5 hours at 68-70°C. After cooling at room temperature, the suspension is filtered through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL), and the NaCl on the filter is washed with methanol. The filtrate is filtered by gravity through a WhatmanTM no. 1 paper in a 500 mL round bottom flask, and the solvent is evaporated under reduced pressure with a rotary evaporator at 50°C. The residue is solubilized with warm distilled water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered under vacuum through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL). The product is washed with water, acetone, and dried. The product is recrystallized with water as the solvent and using charcoal for decolorizing (activated carbon, Norit^r A< 100 mesh, decolorizing). The yield is 76 %.

Synthesis of 1-methyl-2-methoxy-4-imino-6-oxo-dihydropyrimidine (IV)

Compound IV is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-2276) as follows. . To a 250 mL round bottom flask 11g of compound III (77.0 mmol) and 117 mL of 1N NaOH (freshly prepared) are added. The solution is stirred and cooled at 15°C, using a water bath and crushed ice. Then 11.7 mL dimethylsulfate (123.6 mmol) are added dropwise with a pasteur pipette over a period of 60 min. Precipitation eventually occurs upon the addition. The suspension is stirred at 15°C for 3 hours and is left at 4° C overnight. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL). The yield is 38 %.

Synthesis of 1-methyl-4-iminouracil (V)

Compound V is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-2276) as follows. . To a 250 mL round bottom flask 11.26 g of compound IV (72.6 mmol) and 138 mL 12 N HCl are added, and the suspension is stirred at room temperature for 16-20 hours. The suspension is cooled on crushed ice, the product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL). The product is washed with water at 4°C, using a pasteur pipette, until the pH of filtrate is around 4 (about 150 mL). The product is washed with acetone and dried. The yield is 73 %.

Synthesis of 1-methyl-4-imino-5-nitrouracil (VI)

Compound VI is synthesized according to the procedure of Lespagnol et al (Lespagnol, A. et al.(1970) *Chim. Ther.*, 5:321-326) as follows. . To a 250 mL round bottom flask 6.5 g of compound V (46 mmol) and 70 mL of water are added. The suspension is stirred and refluxed at 100°C. A solution of 6.5 g sodium nitrite (93.6 mmol) dissolved in 10 mL water is added gradually to the reaction mixture with a pasteur pipette. Then 48 mL of glacial acetic acid is added with a pasteur pipette. Upon addition, precipitation occurs and the suspension becomes purple. The suspension is stirred and heated for an additional 5 min., and cooled at room temperature and then on crushed ice. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 10-15 ASTM, 60 mL). It is washed with water at 4 °C to remove acetic acid and then with acetone. Last traces of acetic acid and acetone are removed under a high vacuum. The yield is 59 %.

Synthesis of 1-methyl-4,5-diaminouracil (VII)

Compound VII is synthesized by the procedure of Lespagnol et al. (Lespagnol, A. et al.(1970) *Chim. Ther.*, 5:321-326) as follows. To a 100 mL round bottom flask 2 g of compound VI (11.7mmol) and 25 mL water are added. The suspension is stirred and heated in an oil bath at 60°C. Sodium hydrosulfite (88%) is gradually added (40.4 mmol), using a spatula, until the purple color disappears

(approximately 5 g or 24.3 mmol). The suspension is heated for an additional 15 min. The suspension is cooled on crushed ice and left at 4°C overnight. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 30-40 ASTM, 15 mL). The product is washed with water and acetone, and dried. The last traces of acetone are removed under a high vacuum. The yield is 59%.

Synthesis of AAMU-hemisuccinic acid (VIII)

Compound VIII is synthesized as follows. To a 20 mL beaker 0.30 g of compound VII (1.92 mmol) and 5 mL water are added. The suspension is stirred and the pH is adjusted between 8 to 9 with a 3N NaOH solution. Then 0.33 g succinic anhydride (3.3 mmol) is added to the resulting solution, and the mixture is stirred until the succinic anhydride is dissolved. During this process, the pH of the solution is maintained between 8 and 9. The reaction is completed when all the succinic anhydride is dissolved and the pH remains above 8. The hemisuccinate is precipitated by acidification to pH 0.5 with 12N HCl. The product is recovered by filtration on a Whatman™ No. 1 paper, and washed with water to remove HCl. It is then washed with acetone and dried.

Other AAMU or AFMU derivatives

The derivatives shown in Figs. 14 and 15 can also be used for raising antibodies against AAMU or AFMU that can be used for measuring the concentrations of these caffeine metabolites in urine samples.

Synthesis of 1-methylxanthine-8-propionic acid (IX)

This product is synthesized according to a modified procedure of Lespagnol et al. (Lespagnol, A. et al. (1970) *Chim. Ther.*, 5:321-326) as follows. A 0.2 g sample of compound VIII (0.78 mmol) is dissolved in 2-3 mL of a 15% NaOH solution. The resulting solution is stirred at 100°C until all of the solvent is evaporated, and is then maintained at this temperature for an additional 5 min. The resulting solid is cooled at room temperature, and dissolved in 10 mL water. The product is precipitated by acidification to pH 2.8 with 12 N HCl. After cooling at 4°C for 2.5 hours, the product is recovered by filtration on a Whatman™ No. 1 paper, washed with water and acetone, and dried. It is recrystallized from water-methanol (20:80, v/v), using charcoal to decolorize the solution.

Other derivatives of 1X

The other derivatives of 1X, shown in Figs. 16 and 17, can also be used for raising antibodies against 1X and thereby to allow the development of an ELISA for measuring 1X concentration in urine samples.

Synthesis of AAMU

AAMU is synthesized from compound VII according to the procedure of Fink et al (Fink, K. et al. (1964) *J. Biol. Chem.*, 249:4250-4256) as follows. To a 100 mL round bottom flask 1.08 g of compound VII (6.9 mmol) and 20 mL acetic acid anhydride were added. The suspension is stirred and refluxed a 160-165 °C for 6 min. After cooling

at room temperature, the suspension is filtered under vacuum through a sintered glass funnel (Pyrex, 10-15 ASTM, 15 mL). The product is washed with water and acetone, and dried. The product is recrystallized in water.

5

NMR spectroscopy

^1H and ^{13}C NMR spectra of compounds VIII and IX are obtained using a 500 MHz spectrophotometer (VarianTM XL 500 MHz, Varian Analytical Instruments, San Fernando, CA, USA) using deuterated dimethyl sulfoxide as solvent.

10

Conjugation of haptens to bovine serum albumin and rabbit serum albumin

The AAMU-hemisuccinic acid (VIII) and the 1-methylxanthine propionic acid (IX) are conjugated to BSA and RSA according to the following mixed anhydride method. To a 5 mL round bottom flask 31.7 mg of compound VIII (0.12 mmol) or 14.9 mg of compound IX (0.06 mmol) are added. Then 52.2 μL of tri-n-butylamine (0.24 mmol) and 900 μL of dioxane, dried over calcium hydride and freshly distilled, are added. The solution is cooled at 10°C in a water bath using crushed ice. Then 12.6 μL isobutyl chloroformate at 4°C (0.12 mmol, recently purchased or opened) are added and the solution is stirred for 30-40 min at 10-12°C. While the above solution is stirring, a second solution is prepared as follows. In a glass tube 70 mg BSA or RSA (0.001 mmol) are dissolved in 1.83 mL water. Then 1.23 mL dioxane, freshly dried and distilled, is added and the BSA or RSA solution is cooled on ice. After 30-40 min of the above stirring, 70 μL of 1 N NaOH

15

20

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solution cooled on ice is added to the BSA or RSA solution and the resulting solution is poured in one portion to the flask containing the first solution. The solution is stirred at 10-12°C for 3 hours and dialyzed against 1 liter of water for 2 days at room temperature, with water changed twice a day. The protein concentration of the conjugates and the amounts of moles of AAMU or 1X incorporated per mole of BSA or RSA is determined by methods described below. The products are stored as 1 mL aliquots at -20°C

Protein determination by the method of Lowry et al.
(Lowry, O.H. et al. (1951) *J. Biol. Chem.*, 193:265-275)

15 **A) Solutions**

Solution A: 2 g Na_2CO_3 is dissolved in 50 mL water, 10 mL of 10 % SDS and 10 mL 1 NaOH, water is added to 100 mL. Freshly prepared.

Solution B: 1 % NaK Tartrate

20 Solution C: 1 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

Solution D: 1 N phenol (freshly prepared): 3 mL Folin & Ciocalteu's phenol reagent (2.0 N) and 3 mL water.

Solution F: 98 mL Solution A, 1 mL Solution B, 1 mL Solution C. Freshly prepared.

25 BSA: 1 mg/mL. 0.10 g bovine serum albumin (fraction V)/100 mL.

30 **B) Assay**

Standard curve	Tubes # (13 x 100 mm)						
Solution	1	2	3	4	5	6	7

BSA μL)	0	10	15	20	30	40	50
Water μL)	200	190	185	180	170	160	150
Solution F (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0

5 The solutions are vortexed and left 10 min at room temperature.

Solution D μL)	200	200	200	200	200	200	200
----------------------------	-----	-----	-----	-----	-----	-----	-----

The solutions are vortexed and left at room temperature for 1 hour.

10 The absorbance of each solution is read at 750 nm using water as the blank.

Unknown				
Solution	D.F. ^a			Tube # (13 x 100 mm)
	1	2	3	
15 Unknown (μL)	x	x	x	
Water (μL)	y	y	y	
	x + y = 200 μL			
Solution F (mL)	2.0	2.0	2.0	

20 The solutions are vortexed and left 10 min at room temperature.

25 Solution D (μL)	200	200	200
---------------------------------	-----	-----	-----

The solutions are vortexed and left 1 hour at room temperature.

The absorbance of each solution is read at 750 nm using water as the blank.

5 The protein concentration is calculated using the standard curve and taking account of the dilution factor (D.F.).

a. D.F. (dilution factor). It has to be such so that the absorbance of the unknown at 750 nm is within the range of
10 absorbance of the standards.

Method to determine the amounts of moles of AAMU or 1X
15 incorporated per mole of BSA or RSA.

This method gives an approximate estimate. It is a useful one because it allows one to determine whether the coupling proceeded as expected.

20 **A) Solutions**

- 10% sodium dodecyl sulfate (SDS)
- 1% SDS solution
- 0.5 or 1 mg/mL of AAMU-BSA (or AAMU-RSA) in a 1% SDS solution (1 mL).
- 25 - 0.5 or 1 mg/mL of BSA or RSA in a 1% SDS solution (1 mL).

B) Procedure

- The absorbance of the AAMU conjugate solution is
30 measured at 265 nm, with 1% SDS solution as the blank.

- The absorbance of the BSA (or RSA) solution is measured at 265 nm, with 1% SDS solution as the blank.

- The amount of moles of AAMU incorporated per mole of BSA (or RSA) is calculated with this formula:

5

$$y = \frac{A_{265} \text{ (AAMU-BSA)} - A_{265} \text{ (BSA)}}{\epsilon_{265} \text{ (AAMU)} \times [\text{BSA}]}$$

10 Where:

y is the amount of moles of AAMU/mole of BSA (or RSA);
 ϵ_{265} (AAMU) is the extinction coefficient of AAMU = $10^4 \text{ M}^{-1} \text{ cm}^{-1}$; and
 $[\text{BSA}] = \text{BSA (mg/mL)} / 68,000 / \text{mmole}$.

15

To calculate the amount of moles of 1X incorporated per mole of BSA or RSA, the same procedure is used but with this formula:

20

$$y = \frac{A_{252} \text{ (1X-BSA)} - A_{252} \text{ (BSA)}}{\epsilon_{252} \text{ (1X)} \times [\text{BSA}]}$$

Where:

25 y is the amount of moles of 1X/mole of BSA (or RSA);
 ϵ_{252} (AAMU) is the extinction coefficient of 1X = $10^4 \text{ M}^{-1} \text{ cm}^{-1}$; and
 $[\text{BSA}] = \text{BSA (mg/mL)} / 68,000 / \text{mmole}$.

30 **Coupling of haptens to horse radish peroxidase**

The AAMU derivative (VIII) and 1X derivative (IX) are conjugated to horse radish peroxidase (HRP) by the following procedure. To a 5 mL round bottom flask 31.2 mg of compound VIII (or 28.3 mg of compound IX) are added.

Then 500 μ L of dioxane, freshly dried over calcium chloride, are added. The suspension is stirred and cooled at 10°C using a water bath and crushed ice. Then 114 μ L tributylamine and 31 μ L of isobutyl chloroformate (recently opened or purchased) are added. The suspension is stirred for 30 min at 10°C. While the suspension is stirring, a solution is prepared by dissolving 13 mg of horse radish peroxidase (HRP) in 2 mL of water. The solution is cooled at 4°C on crushed ice. After the 30 min stirring, 100 μ L of a 1 N NaOH solution at 4°C is added to the HRP solution and the alkaline HRP solution is poured at once into the 5 mL flask. The suspension is stirred for 4 hours at 10-12°C. The free derivative is separated from the HRP conjugate by filtration through a Sephadex G-25™ column (1.6 x 30 cm) equilibrated and eluted with a 0.05 M sodium phosphate buffer, pH 7.5. The fractions of 1.0-1.2 mL are collected with a fraction collector. During the elution two bands are observed: the HRP conjugate band and a light yellow band behind the HRP conjugate band. The HRP conjugate elutes between fractions 11-16. The fractions containing the HRP conjugate are pooled in a 15 mL tissue culture tube with a screw cap. The HRP conjugate concentration is determined at 403 nm after diluting an aliquot (usually 50 μ L+650 μ L of buffer).

$$[\text{HRP-conjugate}] (\text{mg/mL}) = A_{403} \times 0.4 \times \text{D.F.}$$

The ultraviolet (UV) absorption spectrum is recorded between 320 and 220 nm. The presence of peaks at

264 and 270 nm for AAMU-HRP and 1X-HRP conjugates, respectively, are indicative that the couplings proceeded as expected.

After the above measurements, 5 μ L of a 4 %
5 thiomersal solution is added per mL of the AAMU-HRP or 1X-HRP conjugate solution. The conjugates are stored at 4°C.

Antibody production

Four mature females New Zealand white rabbits
10 (Charles River Canada, St-Constant, Que., Canada) are used for antibody production. The protocol employed in this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. Antibodies of the
15 present invention may be monoclonal or polyclonal antibodies.

An isotonic saline solution (0.6 mL) containing 240 mg of BSA conjugated antigen is emulsified with 0.6 mL of a complete Freund's adjuvant. A 0.5 mL aliquot of the
20 emulsion (100 mg of antigen) is injected per rabbit intramuscularly or subcutaneously. Rabbits are subsequently boosted at intervals of three weeks with 50 mg of antigen emulsified in incomplete Freund's adjuvant. Blood is collected by venipuncture of the ear 10-14 days after
25 boosting. Antisera are stored at 4°C in the presence of 0.01% sodium azide.

Double immunodiffusion in agar plate

An 0.8% agar gel in PBS is prepared in a 60 x 15 mm petri dish. Rabbit serum albumin (100 μ L of 1 mg mL⁻¹) conjugated to AAMU (or 1X) are added to the center well, and 100 μ L of rabbit antiserum are added to the peripheral wells. The immunodiffusion is carried out in a humidified chamber at 37°C overnight and the gel is inspected visually.

Antiserum titers

10 The wells of a microtiter plate are coated with 10 μ g mL⁻¹ of rabbit serum albumin-AAMU (or 1X) conjugate in sodium carbonate buffer, pH 9.6) for 1 hour at 37°C (100 μ L/per well). The wells are then washed three times with 100 μ L TPBS (phosphate buffer saline containing 0.05% Tween
15 TM 20) and unoccupied sites are blocked by an incubation with 100 mL of TPBS containing 0.05% gelatin for 1 hour at 37°C. The wells are washed three times with 100 μ L TPBS and 100 μ L of antiserum diluted in TPBS are added. After 1 hour at 37°C, the wells are washed three times with TPBS, and
20 100 μ L of goat anti-rabbit IgGs-alkaline phosphatase conjugate, diluted in PBS containing 1% BSA, are added. After 1 hour at 37°C, the wells are washed three times with TPBS and three times with water. To the wells are added 100 μ L of a solution containing MgCl₂ (0.5 mM) and p-
25 nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min. at room temperature, the absorbency is read at 405 nm with a microplate reader. The

antibody titer is defined as the dilution required to change the absorbance by one unit (1 au).

Isolation of rabbit IgGs

5 The DE52-cellulose resin is washed three times with sodium phosphate buffer (500 mM, pH 7.50), the fines are removed and the resin is equilibrated with a sodium phosphate buffer (10 mM, pH 7.50). The resin is packed in a 50 x 1.6 cm column and eluted with 200-300 mL
10 equilibrating buffer before use. To antiserum obtained from 50 mL of blood (30-32 mL) is added dropwise 25-27 mL of a 100% saturated ammonium sulfate solution with a Pasteur pipette. The suspension is left at room temperature for 3 h and centrifuged for 30 min. at 2560 g
15 at 20°C. The pellet is dissolved with 15 mL sodium phosphate buffer (10 mM, pH 7.50) and dialyzed at room temperature with the buffer changed twice per day. The dialyzed solution is centrifuged at 2560 g for 10 min. at 20°C to remove precipitate formed during dialysis. The
20 supernatant is applied to the ion-exchange column. Fractions of 7 mL are collected. After application, the column is eluted with the equilibrating buffer until the absorbance at 280 nm becomes less than 0.05 au. The column is then eluted with the equilibrating buffer containing 50
25 mM NaCl. Fractions having absorbencies greater than 0.2 at 280 nm are saved and stored at 4°C. Protein concentrations of the fractions are determined as described above.

Competitive antigen ELISA

Buffers and water without additives are filtered through millipore filters and kept for 1 week. BSA, antibodies, TweenTM 20 and horse radish peroxidase conjugates are added to these buffers and water just prior to use. Urine samples are usually collected 4 hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at -80°C. The urine samples are diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and are subsequently diluted with water to give concentrations of AAMU and 1X no higher than 3×10^{-6} M in the ELISA. All the pipettings are done with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well, 100 µL of a carbonate buffer (100 mM, pH 9.6) containing $2.5 \mu\text{g mL}^{-1}$ antibodies are added to each well. After 90 min. at room temperature, the wells are washed three times with 100 mL of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.05% TweenTM 20.

After the initial wash, unoccupied sites are blocked by incubation for 90 min. at room temperature with 100 µL TBP containing 3% BSA. The wells are washed four times with 100 µL TPB. The washing is followed by additions of 50 µL of 12 mg mL^{-1} AAMU-HRP or 1X-HRP conjugate in 2 x TPB containing 2% BSA, and 50 µL of either water, standard (13 standards; AAMU or 1X, 2×10^{-4} to 2×10^{-8} M) or sample in duplicate. The microplate is gently shaken with an orbital shaker at room temperature for 3-4 hours. The

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wells are washed three times with 100 μ L TPB containing 1% BSA and three times with water containing 0.05% Tween™ 20. To the washed plate is added 150 μ L of a substrate buffer composed of citric acid (25 mM) and sodium phosphate dibasic buffer (50 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% o-phenylenediamine hydrochloride. After 20 min. at room temperature with shaking, the reaction is stopped with 50 μ L of 2.5 M HCl. After shaking the plate 3 min., the absorbances are read with a microtiter plate reader at 490 nm.

Results

Polyclonal antibodies against AAMU and 1X could be successfully raised in rabbits after their conjugation to bovine serum albumin. Each rabbit produced antibody titers of 30,000-100,000 as determined by ELISA. This was also indicated by strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin. On this basis, a) IgGs antibodies were isolated on a DE-52 cellulose column and b) a competitive antigen ELISA for NAT2 phenotyping using caffeine as probe substrate was developed according to the methods described in the above section entitled Materials and Methods.

Contrary to current methods used for phenotyping, the assay involves no extraction, is sensitive and rapid, and can be readily carried out on a routine basis by a technician with a minimum of training in a clinical laboratory.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

5

A competitive antigen ELISA for NAT2 phenotyping using caffeine as a probe substrate

Buffers and water without additives were filtered through millipore filters and kept for 1 week. BSA, antibodies, Tween™ 20 and horse radish peroxidase conjugates were added to these buffers and water just prior to use. Urine samples were usually collected 4 hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at - 80°C. They were diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and were subsequently diluted with water to give concentrations of AAMU and 1X no higher than 3×10^{-6} M in the ELISA. All the pipettings were done with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well, 100 µL of a carbonate buffer (100 mM, pH 9.6) containing $2.5 \mu\text{g mL}^{-1}$ antibodies was pipetted. After 90 min. at room temperature, the wells were washed three times with 100 µL of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.05% Tween™ 20.

After the initial wash, unoccupied sites were blocked by incubation for 90 min. at room temperature with 100 µL TBP containing 3% BSA. The wells were washed four times with 100 µL TPB. This was followed by additions of 50

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μL of 12 mg mL⁻¹ AAMU-HRP or 1X-HRP conjugate in 2 x TPB containing 2% BSA, and 50 μL of either water, standard (13 standards; AAMU or 1X, 2 x 10⁻⁴ to 2 x 10⁻⁸ M) or sample in duplicate. The microplate was gently shaken with an orbital shaker at room temperature for 3-4 hours. The wells were washed three times with 100 μL with TPB containing 1% BSA and three times with water containing 0.05% Tween™ 20. To the washed plate was added 150 μL of a substrate buffer composed of citric acid (25 mM) and sodium phosphate dibasic buffer (50 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% o-phenylenediamine hydrochloride. After 20 min. at room temperature with shaking, the reaction was stopped with 50 μL of 2.5 N HCl. After shaking the plate 3 min., the absorbances were read with a microtiter plate reader at 490 nm.

The competitive antigen ELISA curves of AAMU-Ab and 1X-Ab determinations obtained in duplicate are presented in Fig. 18. Each calibration curve represents the average of two calibration curves. The height of the bars measure the deviations of the absorbency values between the two calibration curves. Data points without bars indicate that deviations of the absorbency values are equal or less than the size of the symbols representing the data points. Under the experimental conditions of the ELISA: background was less than 0.10 au; the practical limits of detection of AAMU and 1X were 2 x 10⁻⁷ M and 2 x 10⁻⁶ M, respectively, concentrations 500 and 50 times lower than those in urine samples from previous phenotyping studies (Kilbane, A.J. et al. (1990) *Clin.*

Pharmacol. Ther., **47**:470-477); the intra-assay and interassay coefficients of variations of AAMU and 1X were 15-20% over the concentration range of 0.01-0.05 mM.

A variety of conditions for the ELISA were tested
5 and a number of noteworthy observations were made:
gelatin, which was used in the competitive antigen ELISA
determination of caffeine in plasma (Fickling, S.A. et al.
(1990) *J. Immunol. Meth.*, **129**:159-164), could not be used
in our ELISA owing to excessive background absorbency
10 which varied between 0.5 and 1.0 au; in the absence of
Tween™ 20, absorbency changes per 15 min. decreased by a
factor of at least 3, and calibration curves were
generally erratic; absorbency coefficients of variation of
samples increased by a factor of 3 to 4 when the
15 conjugates and haptens were added to the wells as a
mixture instead individually.

The cross reactivities of AAMU-Ab and 1X-Ab were
tested using a wide variety of caffeine metabolites and
structural analogs (Table 5 below). AAMU-Ab appeared
20 highly specific for binding AAMU, while 1X-Ab appeared
relatively specific for binding 1X. However, a 11% cross
reactivity was observed with 1-methyluric acid (1U), a
major caffeine metabolite.

Table 5

Cross-reactivity of AAMU-Ab and 1X-Ab towards different caffeine metabolites and structural analogs

5

Compound	% Cross-Reaction	
	AAMU-Ab	1X-Ab
Xanthine	0 ^a	0
Hypoxanthine	0	0
1-Methyl Xanthine (1X)	0	100
3-Methyl Xanthine	0	0
7-Methyl Xanthine	0	0
8-Methyl Xanthine	0	0
1,3-Dimethyl Xanthine (Theophylline)	0	0.2
1,7-Dimethyl Xanthine (Paraxanthine)	0	0.5
3,7-Dimethyl Xanthine (Theobromine)	0	0
1,3,7-Trimethyl Xanthine (Caffeine)	0	0
Uric acid	0	0
1-Methyluric acid	0	11
1,7-Dimethyluric acid	0	0
Guanine	0	0
Uracil	0	0
5-Acetamino-6-amino-uracil	0.6	0
5-Acetamino-6-amino-1-methyluracil (AAMU)	100	0
5-Acetamino-6-amino-1,3-dimethyluracil	0	0

a. The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest compound concentration tested in the ELISA (5×10^{-3} M); concentrations of 5-acetamino-6-amino-1-methyluracil (AAMU) and 1-Methyl Xanthine (1X) required for 50% inhibition in the competitive antigen ELISA were 1.5×10^{-6} M and 10^{-5} M, respectively.

15

The relative high level of cross reactivity of 1U is, however, unlikely to interfere significantly in the

determination of 1X and the assignment of NAT2 phenotypes, since the ratio of 1U:1X is no greater than 2.5:1 in 97% of the population (Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, 49:648-657). This is confirmed by measurements of apparent concentrations of 1X when the ratio varied between 0-8.0 at the fixed 1X concentration of 3×10^{-6} M (Table 6 below). At 1U:1X ratios of 2.5 and 3.0, the apparent increases were 22% and 32%, respectively.

Table 6

The effect of the ratio 1U:1X on the determination of 1X concentration by ELISA at fixed 1X concentration of 3×10^{-6} M

1U:1X ratio	[1X] x 10^6 (M)
0.0	3.00
0.50	2.75
1.00	3.25
1.50	3.25
2.00	3.60
2.50	3.65
3.00	3.95
4.00	4.20
5.00	4.30
6.00	4.50
8.00	4.30

The following observations attested to the validity of the competitive antigen ELISA for NAT2 phenotyping.

- 1) The ELISA assigned the correct phenotype in 29 of 30 individuals that have been phenotyped by capillary electrophoresis (CE) (Lloyd, D. et al. (1992) *J. Chrom.*, 578:283-291).

- 2) In the CE method, the phenotype was determined using AFMU/1X peak height ratios rather than the AAMU/1X molar ratios used in the ELISA. When the molar ratios determined by ELISA and the peak height ratios determined by CE were correlated by regression analysis, the calculated regression equation was $y = 0.48 + 0.87 x$, with a correlation coefficient (r) of 0.84, Taking account that these two ratios are not exactly equal and that Kalow and Tang (Kalow, W. et al. (1993) *Clin. Pharmacol. Ther.*, 53:503-514) have pointed out that using AFMU rather than AAMU can lead to misclassification of NAT2 phenotypes, there is a remarkable agreement between the two methods.
- 3) The ELISA was used in determining the NAT2 phenotype distribution within a group of 146 individuals. Fig. 19 illustrates a histogram of the NAT2 phenotypes of this group as determined by measuring the AAMU/1X ratio in urine samples by ELISA. Assuming an antimode of 1.80, the test population contained 60.4% slow acetylators and 39.6% fast acetylators. This is consistent with previously reported distributions (Kalow, W. et al. (1993) *Clin. Pharmacol. Ther.*, 53:503-514; Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, 47:470-477).

**Determination of 5-acetamino-6-amino-1-methyluracil (AAMU)
and 1-methyl xanthine in urine samples
with the ELISA kit**

5

Table 7

Content of the ELISA kit and conditions of storage

10	Item	Unit	State	Amt	Storage conditions
	Tween™ 20	1 vial	Liquid	250 µL/vial	4°C
15	H ₂ O ₂	1 vial	Liquid	250 µL/vial	4°C
	AAMU-HRP	1 vial	Liquid	250 µL/vial	4°C
	1X-HRP	1 vial	Liquid	250 µL/vial	4°C
20	Buffer A	4 vials	Solid	0.8894 g/vial	4°C
	Buffer B	6 vials	Solid	1.234 g/vial	4°C
25	Buffer C	6 vials	Solid	1.1170 g/vial	4°C
	Buffer D	6 vials	Solid	0.8082 g/vial	4°C
	Plate(AAMU-Ab)	2	Solid	-	4°C
30	Plate (1X-Ab)	2	Solid	-	4°C
	Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
35	Standards (AAMU)	14 vials	Liquid	200 µL	-20°C
	Standards(1X)	14 vials	Liquid	200 µL	-20°C
	1N NaOH	1 bottle	Liquid	15 mL	20°C
40	1N HCl	1 bottle	Liquid	15 mL	20°C

2022-09-20 09:28:04

Conversion of AFMU to AAMU

5 In order to determine the AAMU concentrations in urine samples by competitive antigen ELISA, a transformation of AFMU to AAMU is required.

- Thaw and warm up to room temperature the urine sample.
- Suspend the sample thoroughly with the vortex before
10 pipeting.
- Add 100 μ L of a urine sample to a 1.5 mL-microtube.
- Add 100 μ L of a 1N NaOH solution.
- Leave at room temperature for 10 min.
- Neutralize with 100 μ L 1N HCl solution.
- 15 • Add 700 μ L of Buffer A (dissolve the powder of one vial A/50 mL).

Dilutions of urine samples for the determinations of [AAMU] and [1X] by ELISA

20 The dilutions of urine samples required for determinations of AAMU and 1X are a function of the sensitivity of the competitive antigen ELISA and AAMU and 1X concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X
25 concentrations are about 3×10^{-6} M in the well of the microtiter plate. Generally, dilution factors of 100-400 and 50-100 have been used for AAMU and 1X, respectively.

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Table 8

		Microtube #							
5	Dilution Factor	20x	40x	50x	80x	100x	150x	200x	400x
	Solution	1	2	3	4	5	6	7	8
10	Urine sample(mL) ^a 10 x diluted	500	250	200	125	100	66.7	50	25
	Buffer B (mL)	500	750	800	875	900	933.3	950	975

15 a. Vortex the microtubes containing the urine sample before pipeting.

Store the diluted urine samples at -20°C.

Buffer B: dissolve the content of one vial B/100 mL

20 **Determination of [AAMU] and [1 X] in diluted urine samples by ELISA**

Precautions

25 The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (Substrate buffer). Each sample is determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbance values of duplicates should be within less than 5%. Buffers C, D and E are freshly prepared. Buffer E-H₂O₂ is prepared just prior pipeting in the microtiter plate wells.

30

Preparation of samples:

Prepare Table 9 with a computer and print it. This table shows the content of each well of a 96-well

2025-09-20 09:56:28

microtiter plate. Enter the name of the urine sample (or number) at the corresponding well positions in Table 9. Select the dilution factor (D.F.) of each urine sample and enter at the corresponding position in Table 9. Enter the
5 dilution of each urine sample with buffer B at the corresponding position in Table 9: for example, for a D.F. of 100 (100 μ L of 10x diluted urine sample + 900 μ L buffer B), enter 100/900. See "Dilutions of urine samples..." procedure described above for the preparation of the
10 different dilutions. Prepare the different dilutions of the urine samples in 1.5-mL microtubes. Prepare Table 10 with a computer and print it. Prepare the following 48 microtubes in the order indicated in Table 10.

2025-09-20 09:06:28

Table 9**Positions of blanks, control and urine samples in a microtiter plate**

Sample	Well #	D.F	Dil.	Sample	Well #	D.F	Dil.
Blank	1-2	-		Control	49-50	-	
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72	-	
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96	-	

Table 10
Content of the different microtubes

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	AAMU or 1X	27	9	Dil. Urine
4	S2	AAMU or 1X	28	10	Dil. Urine
5	S3	AAMU or 1X	29	11	Dil. Urine
6	S4	AAMU or 1X	30	12	Dil. Urine
7	S5	AAMU or 1X	31	13	Dil. Urine
8	S6	AAMU or 1X	32	14	Dil. Urine
9	S7	AAMU or 1X	33	15	Dil. Urine
10	S8	AAMU or 1X	34	16	Dil. Urine
11	S9	AAMU or 1X	35	17	Dil. Urine
12	S10	AAMU or 1X	36	Control	Buffer B
13	S11	AAMU or 1X	37	18	Dil. Urine
14	S12	AAMU or 1X	38	19	Dil. Urine
15	S13	AAMU or 1X	39	20	Dil. Urine
16	S14	AAMU or 1X	40	21	Dil. Urine
17	S15	AAMU or 1X	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

Solutions:

5 Buffer C: Dissolve the content of one vial C/50 mL water. Add 25 mL of Tween™ 20.

Buffer D: Dissolve the content of one vial D /25 mL water. Add 25 mL of Tween™ 20.

10 0.05 % Tween™ 20: Add 25 µL of Tween™ 20 to a 100-mL erlenmeyer flask containing 50 mL of water.

2.5 N HCl: 41.75 mL of 12 N HCl/200 mL water. Store in a 250-mL glass bottle.

15 AAMU-HRP conjugate: Add 9 mL of Buffer C to a 15-mL glass test tube. Add 90 µL of AAMU-HRP stock solution.

1X-HRP conjugate: Add 9 mL of a??? Which buffer? 2 % BSA solution to a 15-mL glass test tube. Add 90 µL 1X-HRP stock solution.

20 Buffer E-H₂O₂: Dissolve the content of one vial E-substrate/50 ml water. Add 25 µL of a 30 % H₂O₂ solution (prepared just prior to adding to the microtiter plate wells).

25

Table 11

**Standard solutions of AAMU and 1X
(diluted with buffer B)**

5

	AAMU		1 X	
	Standard	[AAMU]	Standard	[1X]
10	1	1.12×10^{-4} M	1	2.00×10^{-4} M
	2	6.00×10^{-5} M	2	1.12×10^{-4} M
	3	3.56×10^{-5} M	3	6.00×10^{-5} M
	4	2.00×10^{-5} M	4	3.56×10^{-5} M
	5	6.00×10^{-6} M	5	2.00×10^{-5} M
15	6	3.56×10^{-6} M	6	1.12×10^{-5} M
	7	2.00×10^{-6} M	7	6.00×10^{-6} M
	8	1.12×10^{-6} M	8	3.56×10^{-6} M
	9	6.00×10^{-7} M	9	2.00×10^{-6} M
	10	3.56×10^{-7} M	10	1.12×10^{-6} M
20	11	2.00×10^{-7} M	11	6.00×10^{-7} M
	12	1.12×10^{-7} M	12	3.56×10^{-7} M
	13	6.00×10^{-8} M	13	2.00×10^{-7} M
	14	3.56×10^{-8} M	14	1.12×10^{-7} M
	15	2.00×10^{-8} M	15	6.00×10^{-8} M

25 **Conditions of the ELISA**

Add 50 μ L/well of AAMU-HRP (or 1X-HRP) conjugate solution, starting from the last row. Add 50 μ L/well of diluted urine samples in duplicate, standards, blank with a micropipet (0-200 μ L), starting from well # 96 (see Table 9). Cover the plate and mix gently by vortexing for several seconds. Leave the plate at room temperature for 3 h. Wash 3 times with 100 μ L/well with buffer C, using a microtiter plate washer. Wash 3 times with 100 μ L/well with the 0.05% Tween™ 20 solution. Add 150 μ L/well of Buffer E-

- H₂O₂ (prepared just prior adding to the microtiter plate wells). Shake 20-30 min at room temperature with an orbital shaker. Add 50 µL/well of a 2.5 N HCl solution. Shake 3 min with the orbital shaker at room temperature.
- 5 Read the absorbance of the wells with microtiter plate reader at 490 nm. Print the sheet of data and properly identify the data sheet.

10 **Calculation of the [AAMU] and [1X] in urine samples from the data**

- Draw a Table 12 with a computer. Using the data sheet of the microtiter plate reader, enter the average absorbance values of blanks, controls (no free hapten present), standards and samples in Table 12. Draw the
- 15 calibration curve on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma plot (or other plot software). Find the [AAMU] (or [1X]) in the microtiter well of the unknown from the calibration curve and enter the data in Table 13. Multiply
- 20 the [AAMU] (or [1X]) of the unknown by the dilution factor and enter the result in the corresponding case of Table 13.

Table 12**Average absorbance values of samples in the microtiter plate**

Sample	Well #	A₄₉₀	Sample	Well #	A₄₉₀
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

Table 13
AAMU (or 1X) concentrations in urine samples

Sample	D.F.	[AAMU]	[AAMU] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			

Table 14
Composition of the different buffer

	Buffer	pH	Composition	Concen.	[P]
			(mM)	(mM)	
5	A	7.50	0.15629 g/100 mL NaH ₂ PO ₄	11.325	
			1.622 g/100 mL Na ₂ HPO ₄ ·7 H ₂ O	60.099	
10			1.778 g/100 mL (total weight)		
				71.424	
	B	7.50	0.1210191 g /100 mL NaH ₂ PO ₄	8.769	
			1.11309 g /100 mL of Na ₂ HPO ₄ ·7H ₂ O	41.23	
15			1.2341 g/100 mL (total weight)		
				49.999	
	C	7.50	1 g/100 mL of BSA	-	
			0.1210191 g /100 mL of NaH ₂ PO ₄	8.769	
20			1.11309 g /100 mL of Na ₂ HPO ₄ ·7H ₂ O	41.23	
			2.2341 g/100 mL (total weight)		
				49.999	
	D	7.50	2 g/100 mL of BSA		
25			0.1210191 g /100 mL of NaH ₂ PO ₄	8.769	
			1.11309 g /100 mL of Na ₂ HPO ₄ ·7H ₂ O	41.23	
			3.2341 g/100 mL (total weight)		
				49.999	
30	E	5.00	0.52508 g/100 mL of citric acid	25	
			1.34848 g/100 mL of Na ₂ HPO ₄ ·7H ₂ O	50	
			40 mg/100 mL of o-phenylenediamine hydrochloride		
			1.913567 g/100 mL (total weight)		-

35

CYP1A2

Different probe substrates can be used to determine the CYP1A2 phenotype (caffeine, theophylline) In accordance with the present invention suitable probe substrates include without limitation, caffeine, theophylline or acetaminophen.

40

Of these caffeine is the preferred probe substrate. Caffeine is widely consumed and relatively safe. In previous studies the phenotype has been generally determined from the ratios of 1,7-
5 dimethylxanthine (1,7 DMX) + 1,7-dimethyluric acid (1,7 DMU) and 1,3,7-trimethylxanthine (1,3,7 TMX, caffeine). In these studies, the individuals are given an oral dose of a caffeine containing-substance, and the urinary concentrations of the target metabolites determined by
10 HPLC (Kilbane, A. J. et al. (1990) Clin. Pharmacol. Ther 47: 470-477; Tang, B.-K. et al. (1991) Clin. Pharmacol. Ther 49: 648-657) or CE (Meachers et al. (1998) Biomarkers 3: 205-218).

Inhibition of CYP1A2 by quinolone antibiotic
15 agents or serotonin reuptake inhibitors, may result in theophylline toxicity. For these reasons, the utility of a reliable phenotyping test is evident.

Enzyme linked immunosorbent assays (ELISA) have been successfully applied in the determination of low
20 amounts of drugs and other antigenic compounds in plasma and urine samples and are simple to carry out. We have previously developed an ELISA for N-acetyltransferase-2 (NAT2) phenotyping using caffeine as a probe substrate (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) J.
25 Pharm. Biomed. Anal. 13: 1079-1086). We have subsequently tested and proven the validity of the ELISA for the NAT2 phenotyping (Leyland-Jones et al. (1999) Amer. Assoc. Cancer Res. 40: Abstract 356). The ELISA for NAT2 phenotyping is simpler to carry out than the HPLC and CE.

2003-09-20 09:56:30

In accordance with the present invention, there are currently being developed antibodies to measure the molar ratio of caffeine and two caffeine metabolites (1,7-dimethylxanthine (1,7 DMX) and 1,7-dimethyluric acid (1,7 DMU)) in urine samples of an individual collected after caffeine consumption. This ratio provides a determination of an individual's CYP1A2 phenotype. Subsequently, there will be an antigen enzyme linked immunosorbent assay (ELISA) for measuring this ratio using these antibodies. The antibodies of the present invention can be polyclonal or monoclonal antibodies raised against caffeine and two different metabolites of caffeine, which allow the measurement of the molar ratio of caffeine and these metabolites.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the CYP1A2 phenotype of the individual as follows:

$$\frac{1,7\text{-dimethylxanthine (1,7 DMX)} + 1,7\text{-dimethyluric acid (1,7 DMU)}}{\text{caffeine}}$$

Molar ratios of 4 and 12 separate slow, intermediate and fast CYP1A2 metabolizers (Butler et al. (1992) Pharmacogenetics 2: 116-117).

MATERIALS AND METHODS

Materials

N-acetyl-p-aminophenol (acetaminophen), dioxane, formic acid 98-100 % glass redistilled and isobutyl chloroformate are purchased from A&C American Chemicals Ltd. (Ville St-Laurent, Que. Canada); horse radish peroxidase is purchased from Boehringer Mannheim (Montreal, Que.,

Canada); ELISA plates (96-well Easy Wash™ modified flat bottom, high binding; Corning glass wares, Corning, NY, USA) and Falcon 96-well microtest tissue culture plate, no. 3072 (Beckton Dickinson Labware, Franklin, NJ, USA) are purchased from Fisher (Montreal, Quebec, Canada); alkaline phosphatase conjugated to goat anti-rabbit IgGs, Keyhole limpet hemocyanin (KLH) is from Pierce Chemical Co. (Rockford, IL, USA); acetic anhydride, acetonitrile HPLC grade, benzylurea, bovine serum albumin (Cat. No A-3803), N-bromosuccinimide, caffeine metabolites; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride solution (EDAC), ethyl 4-bromobutyrate, ethyl 6-bromohexanoate, methyl cyanoacetate, deuterated chloroform (CDCl₃), deuterated dimethylsulfoxide (d₆), deuterated oxide (D₂O), 1,4-diaminobutane, diethanolamine, dimethylformamide, dimethylsulfate, di-tert-butyl dicarbonate, ethyl chloroformate, Freund's adjuvant (complete and incomplete), glutaraldehyde (50 % v/v), 1-methylxanthine, p-nitrophenolphosphate disodium salt, palladium, 10 wt. % (dry basis) on activated carbon, o-phenylenediamine hydrochloride, polyoxyethylene sorbitan monolaurate (Tween™ 20), porcine skin gelatin, protein A-Sepharose 4B, Sephadex™ G25 fine, sodium hydride, sodium methoxide, theophylline, tributylamine, Tween™ 20, are purchased from Sigma-Aldrich (St-Louis, Missouri, USA); Silica gel particle size 0.040-0.063 mm (230-400 mesh) ASTM Emerck Darmstadt, Germany is purchased from VWR (Montreal, Que., Canada). Dioxane is dried by refluxing over calcium hydride for 4 hours and distilled before use. Other reagents are ACS grade.

5 **Synthetic procedures**

The synthetic routes for the production of caffeine, 1,7-dimethylxanthine, 1,7-dimethyluric acid derivatives are shown in Figs. 20 and 21.

10 **Synthesis of 7-ethoxycarboxypentyl-1,3-dimethylxanthine (II)**

Compound II is synthesized by a procedure similar to that of Daly et al. (Daly, J.W., Mueller, C., Shamin, M. (1991) Pharmacology, 42: 309-321). First, 320 mg of theophylline (I) (1.78 mmole) is dissolved in 7 mL of dry
15 dimethylformamide and then 290 mg of potassium carbonate (2.1 mmole) is added to the reaction mixture. Then, 358 µL of ethyl 6-bromohexanoate (2.02 mmole) is slowly added and the suspension is heated at 60°C for 14 hours. The suspension is filtered in order to remove the potassium
20 carbonate. After washing the potassium carbonate with some dimethylformamide, the solvent is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in chloroform and the solution is dried over magnesium sulfate (MgSO₄). The solvent is
25 evaporated under reduced pressure with a rotary evaporator. 480 mg of the product (slightly yellow oil 1.49 mmole) is obtained, corresponding to a yield of 83.7%.

Synthesis of 7-carboxypentyl-1,3-dimethylxanthine (III)

Compound III is synthesized as follows. First, 225 mg of compound II (0.7 mmole) is dissolved in 7 mL of dimethylformamide. Then, 4 mL of a 10% NaOH solution is added and the solution is refluxed for 30 min (100-125 °C).

5 The solvents are evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in 7 mL of water and the solution is acidified to pH 4 with a 6N HCl solution. Cooling the solution at 4° C crystallizes the product as needle-like crystals. The
10 crystals are filtered under vacuum through a 15-mL sintered glass funnel (10-15 ASTM) and dried. A total of 175 mg of the product is obtained (0.595 mmole), corresponding to a yield of 85%.

15 **Synthesis of 7-methoxycarboxypentyl-1-methylxanthine (V)**

Compound V is synthesized as follows. First, 116 mg of 1-methylxanthine (IV) (0.7 mmole) is dissolved in 4 mL of dimethylformamide. Then, 129 mg of potassium carbonate (0.93 mmole) is added and the resulting solution
20 is stirred. Then, 125 µL of ethyl-6-bromohexanoate (0.7 mmole) in 0.4 mL dimethylformamide is slowly added in three portions. The reaction mixture is heated at 50 °C for 1.5 hours and at 65 °C for 1 hour. After cooling, the suspension is filtered and the filtrate is evaporated
25 under reduced pressure with a rotary evaporator and a high vacuum pump. The product is purified by flash chromatography on a silica gel column (40 x 1 cm) using an ethyl acetate-hexane solution (9:1, v/v) as the eluent.

Synthesis of 7-carboxypentyl-1-methylxanthine (VI)

Compound VI is synthesized as follows. First, 31 mg of compound V (0.1 mmol) is dissolved in 1 mL of dimethylformamide and then 660 μ L of 10% NaOH is added. The
5 resulting solution is refluxed for 30 min (100-120 $^{\circ}$ C). After cooling at room temperature, the solvent is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in water and acidified to pH 4 with a 6N HCl solution. Upon
10 cooling, the solution yields white needle-like crystals, which are filtered and dried. A total of 23 mg of the product (0.082 mmole) is obtained, corresponding to a yield of 82%.

15 Synthesis of 6-amino-1-benzyl uracil (IX)

Compound IX is synthesized according to the procedure similar of that of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. First, 8.64 g of sodium
20 methoxide (160 mmol) is dissolved in 71 mL methanol. The solution is stirred and 7.55 g of benzylurea (50 mmol) and 4.71 mL methyl cyanoacetate (53.4 mmol) are added. The suspension is refluxed 5.5 hours at 68-70 $^{\circ}$ C and cooled at room temperature. After filtration, the methanol is
25 evaporated under reduced pressure with a rotary evaporator. The residue is dissolved in warm distilled water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered

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under vacuum through a sintered glass funnel. The product is washed with water and dried. The yield is 62-65%.

5

Synthesis of 6-amino-1-benzyl-5-bromouracil (X)

Compound X is synthesized according to the procedure of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. First, 3.2 g of 6-amino-1-benzyluracil (15.8 mmol) is dissolved at 100° C in 60 mL acetic acid and 3 mL acetic anhydride. Then, 2.85 g of N-bromosuccinimide (16 mmol) is added in small portions over the next 30 minutes. The reaction mixture is stirred for 1 hour and cooled at room temperature. The precipitate is filtered and washed with small amounts of cold ethanol and dried. A total of 3.36 g of white crystals are obtained (12 mmol), corresponding to a yield of 76%.

20

Synthesis of 6-amino-1-benzyl-5- [N-4'-aminobutyl)-amino] uracil (XI)

Compound XI is synthesized as follows. First, 3 g of compound X (10.71 mmol) is dissolved in 30 mL of 50% 1,4-diaminobutane (bp 158-160°; d 0.877) in water (v/v) and the solution is stirred overnight at room temperature. The solution is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The resulting oil is dissolved in a minimal amount of ethyl acetate-methanol solution (4:1; v/v) and is purified by dry flash

30

chromatography on a silica gel packed in a sintered glass funnel (150 mL) with ethyl acetate-methanol solutions as the eluents. At each successive fraction, the solvent polarity is increased, varying from 60% ethylacetate/40% methanol to 45% ethylacetate/55% methanol (v/v). The product is isolated as a light yellow oil. The amount of purified product obtained is 1.69 g (6.1 mmol), corresponding to a yield of 57%.

10 **Synthesis of 6-amino-1-benzyl-5-[N-4'-tert-butoxycarbonyl-aminol]uracil (XII)**

Compound XII is synthesized as follows. First, 1.63 g of compound XI (5.9 mmol) is dissolved in 5.4 mL of a 1 N NaOH solution. Then, 270 mg of sodium bicarbonate (3.2 mmol) and 2.7 mL of water are added. Then, 5.4 mL of di-tert-butyl dicarbonate solution in isopropanol (1.88 g (8.61 mmol) is dissolved in 5.4 mL isopropanol) is added slowly to the solution of compound XI. After stirring for 3 hours at room temperature, 13.4 mL of water is added and the unreacted di-tert-butyl dicarbonate is extracted twice with 20 mL of petroleum ether. The pH of the reaction mixture is adjusted to 7 by the addition of a 10% citric acid solution and the solution is extracted twice with 40 mL ethyl acetate. The organic layer is dried over sodium sulfate (Na_2SO_4) and is concentrated under reduced pressure with a rotary evaporator. The product is precipitated by the addition of some light petroleum ether to the concentrated solution. The amount of product obtained is 0.99 g of an off-white crystalline compound XII (2.62 mmol), corresponding to a yield of 44%.

5 **Synthesis of 6-amino -1- benzyl-5-[(N-4'tert-butoxy-carbonylaminobutyl-N-ethoxycarbonyl)-amino]-uracil (XIII)**

Compound XIII is synthesized as follows. First, 806 mg of compound XII (2.14 mmol) is suspended in 7.5 mL of water and stirred energetically. Then, 0.5 mL of ethyl
10 chloroformate (5.22 mmol) is added. Then, 3.75 mL of a 1N NaOH solution is added dropwise and the resulting solution is stirred at room temperature for 2.5 hours. The white solid product is filtered, washed thoroughly with water and dried. A total of 741 mg of the product is obtained
15 (1.77 mmol), corresponding to a yield of 82.7%.

20 **Synthesis of 6-amino-1-benzyl-5-[(N-4'tert-butoxy carbonylaminobutyl-N-ethoxycarbonyl)-amino]-3-methyl uracil(XIV)**

Compound XIV is synthesized as follows. First, 712 mg of compound XIII (1.77 mmol) is suspended in 5.8 mL of water. Then, 2.3 mL of a 1N NaOH solution are added and the resulting solution is heated at 40° C and vigorously
25 stirred. Then, 0.23 mL dimethylsulfate (2.43 mmol) is slowly added and the resulting solution stirred at 40 ° C for 1.5 hours. The precipitate, which forms during the reaction, is filtered, washed with water and dried. The product is purified from the precipitate by flash
30 chromatography on a silica gel column (40 x 1cm) using a solution of 4% methanol in dichloromethane as eluent. The product is recrystallized from ethyl acetate. A total of

498 mg of compound XIV (1.15 mmol) is obtained, corresponding to a yield of 65%.

5 **Synthesis of 6-amino-5-[(N-4'tert-butoxycarbonylamino-butyl-N-ethoxycarbonyl)-amino]-3-methyluracil (XV)**

Compound XV is synthesized as follows. First, 440 mg of compound XIV (1.02 mmol) is dissolved in 12 mL methanol and mixed with 252 mg ammonium formate (4 mmol).
10 Then, 240 mg of palladium-on-charcoal (10%) are added under nitrogen atmosphere. The catalytic hydrogenation is performed at room temperature for 3 hours. The catalyst is removed by filtration and the filtrate is evaporated under reduced pressure with a rotary evaporator and a high
15 vacuum pump. A total of 341 mg of the product is obtained (0.99 mmol), corresponding to a yield of 97%.

Synthesis of 7-(4' aminobutyl)-1-methyluric acid (XVI)

20 Compound XVI is synthesized as follows. First, 300 mg of compound XV (0.875 mmol) is dissolved in 4.5 mL dry dimethylformamide and the resulting solution is mixed with 144 mg of sodium hydride (6 mmol). The mixture is stirred at room temperature for 20 min and at 110-115 °C for 30
25 min. The color changes slowly to a dark yellow. After cooling, 6.5 mL of water are added and the solution is acidified to pH 0 with a 6N HCl solution. The solvents are evaporated under reduced pressure with a rotary evaporator and a high vacuum pump, and the crude product is dissolved
30 in a ethyl acetate-methanol solution (1:4, v/v). The inorganic salt is removed by filtration and the yellow

filtrate is purified by flash chromatography on a silica gel column (40 x 1 cm) using a solution of ethyl acetate-methanol (3:7, v/v) as the eluent. The fraction containing the pure product is evaporated under reduced pressure with a rotary evaporator. After titration of the residue with isopropanol, the product is obtained as a pale yellow solid. A total of 98.9 mg of the product is obtained (0.391 mmol), corresponding to a yield of 45%.

10 NMR Spectroscopy

^1H NMR spectra of synthesized are obtained using a 500 MHz spectrophotometer (Varian XL 500 MHz, Varian Analytical Instruments, San Fernando, CA, USA).

15 Coupling of haptens to horse radish peroxidase

The caffeine and 1,7-dimethylxanthine derivatives and the 1,7-dimethyluric acid derivative (after succinylation with succinic anhydride) are conjugated to horse radish peroxidase (HRP) by the following procedure. To a 5 mL round bottom flask 0.12 mmol of the derivative are added. Then, 500 μL of dioxane freshly dried over calcium chloride are added. The suspension is stirred and cooled at 10°C in a water bath using crushed ice. Then, 31 μL isobutylchloroformate (0.24 mmol) (recently opened or purchased) and 114 μL tributylamine (0.47 mmol) are added. The suspension is stirred for 30 min at 10°C . While stirring, a solution is prepared by dissolving 13 mg of horse radish peroxidase (HRP) in 2 mL of water. The solution is cooled at 4°C on crushed ice. After the 30

min. stirring, 100 μ L of a 1 N NaOH solution (freshly prepared) at 4°C is added to the HRP solution and the alkaline HRP solution is poured at once into the 5 mL flask. The suspension is stirred for 4 hours at 10-12°C.

5 The free derivative is separated from the HRP conjugate by filtration on a Sephadex G-25TM fine column (1.6 x 30 cm) equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.0. The fractions of 1.0-1.2 mL are collected manually or with a fraction collector. During elution two

10 bands may be observed: the HRP conjugate and a light yellow band behind the HRP conjugate. The HRP conjugate band eluted between fractions 11-16. The fractions containing the HRP conjugate are pooled in a 15 mL tissue culture with a screw cap. The HRP conjugate concentration

15 is determined at 403 nm after diluting an aliquot (usually 50 μ L + 650 μ L of buffer).

$$[\text{HRP conjugate}] \text{ (mg/mL)} = A_{403} \times 0.4 \times \text{D.F.}$$

20 The ultraviolet (UV) absorption spectrum is recorded between 320 and 220 nm. The presence of additional absorption peaks at 280 nm, 280 nm and 290 nm for caffeine-HRP, 1,7-DMX-HRP and 1,7-DMU-HRP conjugates, respectively, are indicators that the coupling proceeded

25 as expected. After the above measurements, 5 μ L of a 4% thiomersal solution is added per mL of caffeine-HRP, 1,7-DMX-HRP or 1,7-DMU-HRP conjugate solution. The conjugates are stored at 4°C.

Antibody Production

Six mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) are used for antibody production. The protocol employed in this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. An isotonic saline solution (0.6 mL) containing 240 µg of KLH conjugated antigen is emulsified with 0.6 mL of a complete Freund's adjuvant. Then, 0.5 mL of the emulsion (100 µg of antigen) is injected per rabbit intramuscularly or subcutaneously. Rabbits are subsequently boosted at intervals of three weeks with 50 µg of antigen emulsified in incomplete Freund's adjuvant. Blood is collected without anticoagulant in a vacutainer tube by venipuncture of the ear 10-14 days after boosting and kept at 4°C. After clotting, and centrifugation at 4°C, sodium azide is added to the antisera to a final concentration of 0.001% (1µL of a 1 % sodium azide solution per mL of antisera). Antisera are stored as 0.5 mL aliquots at -20 °C.

Antiserum titers

The wells of a microtiter plate are coated with 10 µg mL⁻¹ of bovine serum albumin-caffeine (or 1,7-dimethyl xanthine, 1,7-dimethyluric acid) conjugate in 100 mM sodium carbonate buffer, pH 9.6) overnight at 4° C (150 µL/well). They are then washed three times with TPBS (phosphate buffer saline containing 0.05 % Tween 20) using a Nunc Immuno Wash 12 autoclavable. Unoccupied sites are

blocked by an incubation with 150 μ L/well of TPBS containing 0.05 % porcine gelatin for 2 h at room temperature. The wells are washed three times with TPBS and 150 μ L of antiserum diluted in TPBS are added. After 2
5 h at room temperature, the wells are washed three times with TPBS, and 100 μ L of goat anti-rabbit IgGs-alkaline phosphatase conjugate diluted in PBS containing 1% BSA are added. After 1 h at room temperature, the wells are washed three times with TPBS and three times with water. To the
10 wells are added 150 μ L of a solution containing $MgCl_2$ (0.5 mM) and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min at room temperature, the absorbency is read at 405 nm with a microplate reader. The antibody titer is defined as the
15 dilution required to change the absorbance by one unit (1 au).

Isolation of IgG antibodies

Rabbit IgG antibodies against KLH conjugates are
20 purified by affinity chromatography on a Protein A-Sepharose 4B column as follows. A 0.9 x 15 cm Pharmacia chromatographic column is packed with Protein A-Sepharose 4B suspension to a volume of 1 mL. The column is washed generously with a 0.01 M Na_2HPO_4 - NaH_2PO_4 buffer, pH 8.0
25 containing 0.15 M NaCl (PBS) and then washed with 3-4 mL of a 0.1 M trisodium citrate buffer, pH 3.0. The column is then washed generously with PBS. Then, 1 mL of rabbit antiserum is diluted with 1 mL PBS, and the resulting solution is slowly applied to the column. The column is

washed with 15 mL PBS and eluted with a 0.1 M trisodium citrate buffer, pH 3.0. Three fractions of 2.2 mL are collected in 15-mL graduated tubes containing 0.8 mL of 1 M Tris-HCl buffer, pH 8.5. The purified rabbit IgG anti-
5 bodies are stored at 4°C in the presence at 0.01 % sodium azide.

Competitive antigen ELISA

Buffers and water without additives are filtered
10 through 0.45 µm millipore filters and kept for one week, except the substrate buffer which is freshly prepared. BSA, antibodies, Tween™ 20 and horse radish peroxidase are added to buffers and water just prior to use. Urine samples are usually collected four hours after drinking a
15 cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at -20°C as 1-mL aliquots in 1.5-mL microtubes. For the ELISA, the urine samples are diluted with isotonic sodium phosphate buffer, pH 7.5 (310 mosM) to give concentrations of caffeine, 1,7-DMX and 1,7-
20 DMU no higher than 3×10^{-6} M in the microtiter plate wells. Wells of the ELISA plate are washed with a Nunc-Immuno wash 12 washer. Sixteen milliliters of a solution of 6.6 µg ml⁻¹ of isolated IgG antibodies is prepared in a
25 100 mM sodium carbonate buffer, pH 9.6, and 150 µL of this solution is pipetted in each well of a microtiter plate using a eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips from Brinkmann). After coating the wells with antibodies at 4°C for 20 hours, the wells are washed 3 times with the isotonic sodium

phosphate buffer containing 0.05% TweenTM 20 (IPBT) and properly drained by inverting the plate and absorbing the liquid on to a piece of paper towel. Thirty milliliters of a solution of a IPBT solution containing 1% BSA is prepared and 150 μ L of this solution is pipetted in each well using a eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and 200 μ L yellow tips (Sarstedt yellow tips for P200 Gilson Pipetman). After 3 hours at room temperature, the wells are washed 3 times with IPBT solution and drained. Samples of 400 μ L for determination of caffeine, 1,7-DMX and 1,7-DMU are prepared in 1.5-mL microtubes using Sarstedt yellow tips and a P200 Gilson Pipetman. Then, 200 μ L of each sample are pipetted in duplicate in a Falcon 96-well microtest tissue culture plate according to the pattern shown in Figure 22, using Sarstedt yellow tips and a P200 Gilson Pipetman. Using an eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and changing the tips of the eight channel pipet (200 μ L Flex tips from Brinkmann) at each row, 150 μ L of samples are transferred in the corresponding wells of a 96-well ELISA microtiter plate coated with antibodies. After the addition of the samples, the microtiter plates are covered and left standing at room temperature for 2 h. While the plate is left standing the substrate buffer without the hydrogen peroxide and o-phenylenediamine hydrochloride is prepared (25 mM citric acid and 50 mM sodium phosphate dibasic buffer, pH 5.0). The microtiter plate is washed 3 times with the IPBT solution and 3 times with a 0.05% TweenTM 20 solution and drained. Then, 50 μ L of hydrogen

peroxide and 40 mg of o-phenylenediamine are added to the substrate buffer. One hundred fifty microliters (150 μ L) of the substrate buffer solution is then added to each well using a eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and 200 μ L Flex tips (Brinkmann). The microtiter plate is covered and shaken for 25-30 min at room temperature and the enzymatic reaction is stopped by adding 50 μ L/well of a 2.5 N HCl solution using an eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and 200 μ L Flex tips (Brinkmann). After gently shaking for 3 min., the absorbance is read at 490 nm with a microplate reader.

Standard solutions of Caffeine, 1,7-DMX and 1,7-Dimethyluric acid solutions for ELISA

A 100 mL stock solution of each of caffeine, 1,7-DMX and 1,7-DMU acid at concentrations of 6.00×10^{-4} M, in the 310 mosM sodium phosphate buffer, pH 7.5 (IPB) is prepared in a 100 mL volumetric flask. The solution is stirred to insure complete solubilization.

The stock solutions are stored as 1 mL aliquots at -20°C .

On the day of the ELISA, one aliquot is thawed and warmed up to room temperature.

The standard solutions of the above compounds are prepared as outlined in Table 15 below.

TABLE 15**Standard Solutions**

Standard #	[Compound]	Composition
1	6.00×10^{-4} M	Stock solution
2	2.00×10^{-4} M	200 μ L S1 + 400 μ L IPB
3	1.12×10^{-4} M	200 μ L S1 + 868 μ L IPB
4	6.00×10^{-5} M	100 μ L S1 + 900 μ L IPB
5	3.56×10^{-5} M	60 μ L S1 + 951 μ L IPB
6	2.00×10^{-5} M	100 μ L S2 + 900 μ L IPB
7	1.12×10^{-5} M	100 μ L S3 + 900 μ L IPB
8	6.00×10^{-6} M	100 μ L S4 + 900 μ L IPB
9	3.56×10^{-6} M	100 μ L S5 + 900 μ L IPB
10	2.00×10^{-6} M	100 μ L S6 + 900 μ L IPB
11	1.12×10^{-6} M	100 μ L S7 + 900 μ L IPB
12	6.00×10^{-7} M	100 μ L S8 + 900 μ L IPB
13	3.56×10^{-7} M	100 μ L S9 + 900 μ L IPB
14	2.00×10^{-7} M	100 μ L S10 + 900 μ L IPB
15	1.12×10^{-7} M	100 μ L S11 + 900 μ L IPB
16	6.00×10^{-8} M	100 μ L S12 + 900 μ L IPB
17	3.56×10^{-8} M	100 μ L S13 + 900 μ L IPB
18	2.00×10^{-8} M	100 μ L S14 + 900 μ L IPB
19	2.00×10^{-9} M	100 μ L S18 + 900 μ L IPB
20	2.00×10^{-10} M	100 μ L S19 + 900 μ L IPB
21	2.00×10^{-11} M	100 μ L S20 + 900 μ L IPB
22	2.00×10^{-12} M	100 μ L S21 + 900 μ L IPB
23	2.00×10^{-13} M	100 μ L S22 + 900 μ L IPB

Antibody Specificity

To ensure accuracy in the ELISA measurement of CYP1A2 phenotyping, the antibodies must have specificity
5 for their individual caffeine metabolites, with little or no recognition of other derivatives. To ensure their selectivity an ELISA is performed with standard solutions of the compounds listed in Table 16. An ideal antibody specificity result is hypothesized with the Table 16 as
10 well.

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Table 16

Cross-reactivity of caffeine-Ab, 1,7-DMX-Ab and 1,7-DMU-Ab towards caffeine metabolites and structural analogs

5

% Cross-reaction			
Compound	Caffeine-Ab	1,7-DMX-Ab	1,7-DMU-Ab
Caffeine	100	0 ^a	0
<u>Xanthine</u>	<u>0</u>	<u>0</u>	<u>0</u>
Hypoxanthine	0	0	0
1-Methyl Xanthine	0	0	0
3-Methyl Xanthine	0	0	0
7-Methyl Xanthine	0	0	0
8-Methyl Xanthine	0	0	0
1,3-Dimethyl Xanthine ^b	0	0	0
1,7-Dimethyl Xanthine ^c	0	100	0
3,7-Dimethyl Xanthine ^d	0	0	0
Uric acid	0	0	0
1-Methyluric acid	0	0	0
3-Methyluric acid	0	0	0
7-Methyluric acid	0	0	0
1,3-Dimethyluric acid	0	0	0
1,7-Dimethyluric acid	0	0	100
3,7-Dimethyluric acid	0	0	0
1,3,7-Trimethyluric acid	0	0	0
Guanine	0	0	0
Uracil	0	0	0
AAU ^e	0	0	0
AAMU ^f	0	0	0
AADMU ^g	0	0	0

a. The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest concentration tested in the ELISA (5×10^{-3} M); concentrations of caffeine, 1,7-Dimethyl Xanthine and 1,7-Dimethyluric acid required for 50% inhibition in the competitive antigen ELISA will be determined; b, 1,3-Dimethyl Xanthine, theophylline; c, 1,7-Dimethyl Xanthine, paraxanthine; d, 3,7-Dimethyl Xanthine, theobromine; e, AAU, 5-acetamido-6-aminouracil; f, AAMU, 5-acetamido-6-amino-3-methyluracil; g, AADMU, 5-acetamido-6-amino-1,3-dimethylxanthine.

RESULTS

Positive creation of antibodies against caffeine, 1,7-DMX, and 1,7-DMU can be seen by antibody titers of 30,000-100,000 as determined by the ELISA, strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin, and low cross-reactivity with other caffeine derivatives. These results constitute positive conditions for the development of a competitive antigen ELISA according to the methods described in the above section entitled Materials and Methods.

In accordance with one embodiment of the present invention, a competitive antigen ELISA is developed for CYP1A2 phenotyping using caffeine as the probe substrate. Contrary to current methods used for phenotyping, the assay is sensitive, rapid and can be readily carried out on a routine basis by a technician with a minimum of training in a clinical laboratory.

EXAMPLE II

Determination of Caffeine, 1,7-Dimethyl Xanthine (1,7-DMX) and 1,7-Dimethyluric acid (1,7-DMU) in urine samples with the ELISA kit

5

Table 17

Content of the ELISA kit and conditions of storage

Item	Unit	State	Amt.	Storage Conditions
Tween™ 20	1 vial	liquid	250 µL/vial	4°C
H ₂ O ₂	1 vial	liquid	250 µL/vial	4°C
Caffeine-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMX-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMU-HRP	1 vial	liquid	250 µL/vial	4°C
Buffer A	4 vials	Solid	0.8894 g /vial	4°C
Buffer B	6 vials	Solid	1.234 g/vial	4°C
Buffer C	6 vials	Solid	1.1170 g/vial	4°C
Buffer D	6 vials	Solid	0.8082 g/vial	4°C
Plate (Caffeine-Ab)	2	Solid	-	4°C
Plate (1,7-DMX-Ab)	2	Solid	-	4°C
Plate (1,7-DMU-Ab)	2	Solid	-	4°C
Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
Standards (Caffeine)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMX)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMU)	14 vials	Liquid	200 µL	-20°C
1N NaOH	1 bottle	Liquid	15 mL	20°C
1N HCl	1 bottle	Liquid	15 mL	20°C

Dilutions of urine samples for the determinations of

[Caffeine], [1,7-DMX] and [1X] by ELISA

10

The dilutions of urine samples required for determinations of caffeine, 1,7-DMX and 1,7-DMU are a function of the sensitivity of the competitive antigen ELISA and of caffeine, 1,7-DMX and 1,7-DMU concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X are about 3×10^{-6} M in the well of the microtiter plate.

Table 18

Dilution Factor	Microtube #							
	20x	40x	50x	80x	100x	150x	200x	400x
Solution	1	2	3	4	5	6	7	8
Urine Sample (mL) ^a	500	250	200	125	100	66.7	50	25
10x diluted								
Buffer B (mL)	500	750	800	875	900	933.3	950	975

a: The microtubes containing the urine sample are vortexed before pipetting.

The diluted urine samples are stored at -20°C in a box for microtubes.

Buffer B: dissolve the content of 1 vial B/ 100 mL water

Determination of [caffeine], [1,7-DMX] and [1,7-DMU] in diluted urine samples by ELISA

Precautions

The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (substrate buffer). Each sample is determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbency values of duplicates should be within less than 5%. Buffers C, D, E are freshly prepared. Buffer E-H₂O₂ is

prepared just prior to pipeting in the microtiter plate wells.

Preparation of samples:

- 5 Prepare Table 19 with a computer and print it. This table shows the contents of each well of a 96 well microtiter plate. Enter the name of the urine sample (or number) at the corresponding well positions in Table 19. Select the dilution factor (D.F.) of each urine sample and enter at the corresponding position in Table 19. Enter the dilution of each urine sample with buffer B at the corresponding position in Table 19: for example a D.F. of 100 (100 μ L of 10x diluted urine sample + 900 μ L buffer B), enter 100/900. See "Dilutions of urine samples..." procedure described above for the preparation of the different dilutions. Prepare the different dilutions of the urine samples in 1.5 mL microtubes using a styrofoam support for 100 microtubes. Prepare Table 20 with a computer and print it. Using a styrofoam support (100 microtubes), prepare the following 48 microtubes in the order indicated in Table 20.

Table 19**Positions of blanks, control and urine samples in a microtiter plate**

Sample	Well #	D.F.	Dil.	Sample	Well #	D.F.	Dil.
Blank	1-2	-		Control	49-50		-
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72		-
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96		-

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Table 20
Content of the different microtubes

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	Caffeine/1,7-DMX/1,7-DMU	27	9	Dil. Urine
4	S2	Caffeine/1,7-DMX/1,7-DMU	28	10	Dil. Urine
5	S3	Caffeine/1,7-DMX/1,7-DMU	29	11	Dil. Urine
6	S4	Caffeine/1,7-DMX/1,7-DMU	30	12	Dil. Urine
7	S5	Caffeine/1,7-DMX/1,7-DMU	31	13	Dil. Urine
8	S6	Caffeine/1,7-DMX/1,7-DMU	32	14	Dil. Urine
9	S7	Caffeine/1,7-DMX/1,7-DMU	33	15	Dil. Urine
10	S8	Caffeine/1,7-DMX/1,7-DMU	34	16	Dil. Urine
11	S9	Caffeine/1,7-DMX/1,7-DMU	35	17	Dil. Urine
12	S10	Caffeine/1,7-DMX/1,7-DMU	36	Control	Buffer B
13	S11	Caffeine/1,7-DMX/1,7-DMU	37	18	Dil. Urine
14	S12	Caffeine/1,7-DMX/1,7-DMU	38	19	Dil. Urine
15	S13	Caffeine/1,7-DMX/1,7-DMU	39	20	Dil. Urine
16	S14	Caffeine/1,7-DMX/1,7-DMU	40	21	Dil. Urine
17	S15	Caffeine/1,7-DMX/1,7-DMU	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

Solutions

- 5 Buffer C: Dissolve the content of one vial C/50 mL water.
Add 25 mL of Tween™ 20.

- Buffer D: Dissolve the content of one vial D/25 mL water.
Add 25 mL of Tween™ 20.
- 5 0.05% Tween™ 20: Add 25 mL of Tween™ 20 in a 100 mL erlenmeyer flask containing 50 mL of water.
- 2.5N HCl: 41.75 mL of 12N HCl/200 mL water.
Store in a 250 mL glass bottle.
- 10 Caffeine-HRP conjugate: Add 9 mL of Buffer C in a 15 mL glass test tube. Add 90 µL of caffeine-HRP stock solution.
- 1,7-DMX-HRP conjugate: Add 9 mL of Buffer C in a 15 mL glass test tube. Add 90 µL of 1,7-DMX-HRP stock solution.
- 15 1,7-DMU-HRP conjugate: Add 9 mL of the 2% BSA solution in a 15 mL glass test tube. Add 90 µL of 1,7-DMU-HRP stock solution.
- 20 Buffer E - H₂O₂: Dissolve the contents of 1 vial E-substrate/50 mL water. Add 25 µL of a 30% H₂O₂ solution (prepared fresh).

Table 21**Standard solutions of caffeine, 1,7-DMX and 1,7-DMU (diluted with buffer B)**

Standard	Caffeine	Standard	1,7-DMX	Standard	1,7-DMU
1	1.12×10^{-4} M	1	1.12×10^{-4} M	1	1.12×10^{-4} M
2	6.00×10^{-5} M	2	6.00×10^{-5} M	2	6.00×10^{-5} M
3	3.56×10^{-5} M	3	3.56×10^{-5} M	3	3.56×10^{-5} M
4	2.00×10^{-5} M	4	2.00×10^{-5} M	4	2.00×10^{-5} M
5	6.00×10^{-6} M	5	6.00×10^{-6} M	5	6.00×10^{-6} M
6	3.56×10^{-6} M	6	3.56×10^{-6} M	6	3.56×10^{-6} M
7	2.00×10^{-6} M	7	2.00×10^{-6} M	7	2.00×10^{-6} M
8	1.12×10^{-6} M	8	1.12×10^{-6} M	8	1.12×10^{-6} M
9	6.00×10^{-7} M	9	6.00×10^{-7} M	9	6.00×10^{-7} M
10	3.56×10^{-7} M	10	3.56×10^{-7} M	10	3.56×10^{-7} M
11	2.00×10^{-7} M	11	2.00×10^{-7} M	11	2.00×10^{-7} M
12	1.12×10^{-7} M	12	1.12×10^{-7} M	12	1.12×10^{-7} M
13	6.00×10^{-8} M	13	6.00×10^{-8} M	13	6.00×10^{-8} M
14	3.56×10^{-8} M	14	3.56×10^{-8} M	14	3.56×10^{-8} M
15	2.00×10^{-8} M	15	2.00×10^{-8} M	15	2.00×10^{-8} M

Conditions of the ELISA

- 5 Add 50 μ L/well of Caffeine-HRP (1,7-DMX-HRP or 1,7-DMU-HRP) conjugate solution starting from the last row. Add 50 μ L/well of diluted urine samples in duplicate, standards, blank with a micropipet (0-200 μ L), starting from well # 96 (see Table 22). The plate is covered and
- 10 mixed gently by vortexing for several seconds. The plate is left at room temperature for 3 hours. The plate is then washed three times with 100 μ L/well buffer C, using a microtiter plate washer. The plate is then washed 3 times with 100 μ L/well 0.05% TweenTM 20 solution. Add 150 μ L/well
- 15 of Buffer E - H₂O₂ (prepared just prior to pipeting in the

microtiter plate wells). The plate is shaken for 20-30 min. at room temperature using an orbital shaker. Add 50 μ L/well of a 2.5N HCl solution. The plate is shaken 3 min. with the orbital shaker at room temperature. The
5 absorbance of the wells is read with a microtiter plate reader at 490 nm. Print the sheet of data and properly label.

10 **Calculation of the [caffeine], [1,7-DMX] and [1,7-DMU] in urine samples from the data**

Draw Table 22 with a computer. Using the data sheet of the microtiter plate reader, enter the average absorbance values of blanks, controls (no free hapten present), standards and samples in Table 22. Draw the
15 calibration curve on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma-plot (or other plot software). Find the [AAMU] (or [1X]) in the microtiter well of the unknowns from the calibration curve and enter the data in Table 23. Multiply
20 the [caffeine] ([1,7-DMX] or [1,7-DMU] of the unknown by the dilution factor and enter the result in the corresponding cell of Table 23.

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Table 22

Average absorbance values of samples in the microtiter plate

Sample	Well #	A ₄₉₀	Sample	Well #	A ₄₉₀
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

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Table 23**Caffeine, 1,7-DMX and 1,7-DMU concentrations in urine samples**

Sample	D.F.	[Caffeine]	[caffeine] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			

Table 24
Composition of the different buffers

Buffer	pH	Composition	Conc. (mM)	[P] (mM)
A	7.50	0.15629 g/100 mL NaH ₂ PO ₄	11.325	71.424
		1.622 g/100 mL Na ₂ HPO ₄ ·7H ₂ O	60.099	
		1.778 g/100 mL (total weight)		
B	7.50	0.1210191 g/100 mL NaH ₂ PO ₄	8.769	49.999
		1.11309 g/100 mL Na ₂ HPO ₄ ·7H ₂ O	41.23	
		1.2341 g/100 mL (total weight)		
C	7.50	1 g/ 100mL BSA		49.999
		0.1210191 g/100 mL NaH ₂ PO ₄	8.769	
		1.11309 g/100 mL Na ₂ HPO ₄ ·7H ₂ O	41.23	
		2.2341 g/100 mL (total weight)		
D	7.50	2 g/ 100mL BSA		49.999
		0.1210191 g/100 mL NaH ₂ PO ₄	8.769	
		1.11309 g/100 mL Na ₂ HPO ₄ ·7H ₂ O	41.23	
		3.2341 g/100 mL (total weight)		
E	5.00	0.52508 g/ 100mL of citric acid	25	-
		1.34848 g/100 mL Na ₂ HPO ₄ ·7H ₂ O	50	
		40 mg/100 mL of o-phenylenediamine hydrochloride		
		1.913567 g/100 mL (total weight)		

5 Accordingly, an ELISA system is provided that is specific for at least NAT2. Alternatively, the ELISA protocol outlined hereinabove, may be adapted for a plurality of enzymes of interest. Fig. 17 exemplifies a multi-determinant assay according to an embodiment of the present invention. Furtherstill, a multi-determinant

10

assay of the present invention may provide more than one 6 X 6 array, as illustrated in Fig. 17, in each well of a standard microplate. Preferably, each well will be provided with 4 6 x 6 arrays according to this aspect of the present invention.

The single or multi-determinant assay system of the present invention include(s) metabolite-specific binding agents for the detection of drug-specific metabolites in a biological sample. Such binding agents are preferably antibodies and the assay system is preferably an ELISA, as exemplified in the cases of NAT2 discussed herein above. A detection method according to an embodiment of the present invention is exemplified in Fig. 18. An assay system of the present invention is exemplified in Fig. 19 and provides means to detect metabolites specific to the metabolic pathway(s) used to metabolize amonafide.

The present invention provides a convenient and effective tool for use in both a clinical and laboratory environment. The present invention is particularly suited for use by a physician in a clinic, whereby phenotypic determinants for at least NAT2 can be quickly and easily obtained. According to an embodiment of the present invention, a ready-to-use kit is provided for fast and accurate determination of at least NAT2 determinants. The assay system and kit preferably employ antibodies specific to a plurality of metabolites on a suitable substrate allowing for detection of the preferred metabolites in a biological sample of an individual after consumption of a corresponding probe drug. In accordance with a preferred

embodiment of the present invention, the kit of the present invention will provide means to determine metabolic determinants for at least N-acetyltransferase-2 (NAT-2). Alternatively, the kit of the present invention will provide means for N-acetyltransferase-2 (NAT-2) and at least one of the following enzymes, CYP1A2, N-acetyltransferase-1 (NAT-1), CYP2D6, CYP2A6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19. The assay system of the present invention may be provided in a plurality of forms as known in the art, including but not limited to an ELISA assay, a high-throughput ELISA assay or a dipstick based ELISA assay.

EXAMPLE II

Use of Metabolic Phenotyping in Determining Individualized Treatment Regimes with Amonafide

The exposure of an individual to a drug is described by the concept of area-under-the curve (commonly referred to as AUC). AUC is related to clearance by the following equation:

$$\text{AUC} = \text{dose} / \text{clearance}$$

Thus, if an individual's clearance is known, the dose can be individualized to achieve a desired AUC by the equation:

$$\text{Dose} = \text{desired AUC} \times \text{clearance}$$

An individual's rate of drug clearance is important as it determines the circulating drug concentrations. Both efficacy and toxicity are

determined, in part, by the circulating concentrations of drug

Therefore, to individualize therapy a model is developed encompassing the numerous factors, which could possibly play a role in an individual's clearance value for a particular medication(s) and hence predict a dose with maximal efficacy and minimal toxicity. As drug metabolism is the principal determinant of circulating drug concentrations, determining an individual's rate of drug metabolism is an important factor for the development of a successful model for the individualization of therapy. The model of the present invention will account for an individual's rate of NAT2 metabolism in determining a specific dose of amonafide for that individual.

Other factors can alter drug clearance, such as body surface area, hepatic enzyme and protein levels (including serum alanine aminotransferases (ALT), albumin, alkaline phosphatases and serum α -1-acidicglycoprotein (AAG)), and drug transport proteins (including P-glycoprotein (pgp)).

Other individual specific characteristics may play a role in determining individual dose-limiting toxicity. According to another aspect of the present invention, other influencing factors may be accounted for, in addition to the rate of metabolism, in the model for the individualization of therapy with amonafide. For example, in the case of many chemotherapeutic drugs, myelosuppression is the dose-limiting toxicity, and hence

an individual's pretreatment white blood cell (WBC) count could be an important factor in predicting toxicity.

Using multivariate analysis these individual factors will be examined for correlation to efficacy and toxicity. In accordance with one embodiment of the present invention, factors identified as having a significant correlation to either efficacy or toxicity will be included in the model along with drug metabolism.

The importance of drug metabolism in determining an individual's rate of drug clearance renders it as the most important factor in determining the efficacy and toxicity of many drugs. Some of the metabolic enzymes mentioned in the context of this invention have a clear bimodal distribution of metabolism, allowing the separation of the population into poor and extensive metabolizers. However, within each phenotypic group there is a wide variation in metabolic rates. It may be a naïve to regard all individuals with metabolic ratios greater than a predetermined cut off value as being equivalent. This attempt to classify the population in two or three phenotypic groups is even more difficult for enzymes without a bimodal distribution. The classification of individuals into this limited classification may not allow for the complete exploitation of an individual's pattern of metabolism. In some cases this simple classification is sufficient. For example, some individuals may have an enzyme specific deficiency, such as CYP2D6 and as a result are at risk for severe complications if high doses of a particular drug, such as ProzacTM are prescribed. However,

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this simple classification would not allow for differential dosing of the extensive metabolizers as a function of the molar ratio calculated during determination of phenotype. If the simple classification of extensive CYP2D6 metabolizers was used, all individuals with a molar ratio of >0.3 (dextromethorphan as probe drug) would receive the same dose. We are proposing the development of a dosing scale that would produce an increasing dose with increasing metabolic ratio, as exemplified in Fig. 20. If only the bimodal distribution is considered, only two possible doses can be prescribed. Accordingly, the individualization of therapy with amonafide is proposed in accordance with the present invention. As a result, the categorical treatment with amonafide based on phenotype will be replaced with individualization of treatment whereby the metabolism of each individual is assessed on an individual basis and a corresponding individual dosage is determined. In this manner, amonafide is prescribed on an individual basis in dosages corresponding with an individual's phenotypic ability for metabolism.

In some cases multiple enzymes play key roles in determining the rate of drug metabolism. Therefore, the monitoring of only one metabolic enzyme in such cases may not provide complete information for individualizing therapy. The use of a multi-determinant assay examines multiple enzymes to provide additional metabolism-related information thereby providing a more accurate model for individualizing therapy is generated. For example, preliminary studies have suggested that amonafide may also

be a substrate of the cytochrome P450 enzyme, CYP1A2, and that the metabolism of amonafide by CYP1A2 is inhibited by N-acetylamonafide. Therefore, the use of a multi-determinant assay, which measures the rates of CYP1A2 and NAT2 metabolism, may provide a more accurate model.

Individuals with extreme metabolic phenotypes are often at high risks for either toxicity or inefficacy of therapy. These ultraextensive or extremely poor metabolizers can often be identified by genotyping. For several metabolic enzymes genetic polymorphisms exist which result in an enzyme deficiency or the production enzyme with null activity. These individuals will not be affected by enzyme inducers or inhibitors and will consistently be extremely poor metabolizers. Identifying those individuals who carry these genetic polymorphisms allows physicians to avoid prescribing a drug metabolized by the enzyme in question. Conversely, several genetic polymorphisms have been identified that result in high levels of enzyme and/or increased enzyme activity. In addition, some individuals have been identified with multiple copies of the gene containing the polymorphism. As for the extremely poor metabolizers, these individuals may be excluded from certain treatment regimes due to increased risk of toxicity or lack of response.

Therefore, the use of genotyping to identify which individuals should be treated with a particular drug may be an excellent precursor to individualizing the individual's therapy based upon their specific phenotype. In doing so, an individual having a specific allelic

variation corresponding to an enzyme specific inefficiency in metabolism can be identified before undergoing preliminary phenotyping procedures and treatment with a probe drug or substrate.

5 The knowledge of an individual's (multiple) phenotypic profile will allow physicians to:

- 1) determine if the individual has a phenotype that allows for the safe prescription of a drug;
- 10 2) determine the optimal drug dose in terms of drug efficiency and drug safety for an individual;
- 15 3) determine which drug of a plurality of drugs used for treating an individual's pathology or condition is the optimal drug in terms of drug efficiency and drug safety for that individual.

 The knowledge of an individual's phenotypic profile for one or more enzymes will allow for the detection of
20 drug(s) that could cause significant side effects or be inefficient in individuals with a specific phenotypic profile. In addition, the phenotypic profile will allow the development of an individualized dosing scheme with dose related to level of enzyme activities. The
25 implementation of the multi-determinant phenotyping profile in treatment and dosing selection will lead to a marked decrease in side effects and increase in therapeutic efficiency.

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Amonafide

Amonafide has been shown to have antitumor activity in non-small cell lung cancer, prostate cancer and breast cancer. The most common dose-limiting side effect of amonafide has been granulocytopenia. Amonafide is metabolized by NAT2, forming its major metabolite N-acetylamonafide. Fig. 21 depicts the structures of amonafide and N-acetylamonafide.

Previous studies have concluded that fast NAT2 acetylators would be expected to experience the most severe toxicity and slow acetylators may be significantly underdosed [Ratain et al. J Clin Oncol 13:741-47, 1995]. One study has generated a dosing recommendation of 250 and 375mg/m² for fast and slow acetylators, respectively [Ratain et al. (1993) Cancer Res. 53:2304-2308]. This categorization of therapy is limited by several factors. While the categorical segregation of individuals into slow and fast acetylators may produce an improvement in the levels of toxicity and response rates, it is not optimal as this approach does not account for individual phenotypic differences. To determine the rate of NAT2 activity, caffeine is used as a probe substrate and the molar ratio of the caffeine metabolites AAMU and 1X is calculated. To segregate the population into slow and fast acetylators a value of 1.80 was used (<1.80=slow acetylators, >1.80=fast acetylators). However, for fast acetylators the range of molar ratios can vary from just above 1.80 up to 12. Therefore, it may be naïve to assign a single dose to all fast acetylators. A more appropriate method is the incorporation of the individual's specific

molar ratio as a component of the individualization of therapy model.

An additional limiting factor in the categorical methodology mentioned above, is the necessity of measuring
5 N-acetylamonafide levels at 24 hours post treatment. This measurement is clinically unpractical as it requires an additional test specific to amonafide, and the collection of an addition serum sample.

The present invention provides for an
10 individualization model based upon at least an individual's specific NAT2 phenotype for use in the individualization of therapy with amonafide. The individualization model of the present invention may further include other enzyme-specific determinants as well
15 as other factors, which have a significant contribution to amonafide clearance (including hepatic enzyme levels), or toxicity (including pretreatment WBC). This model will not segregate individuals into fast or slow phenotypic classifications and treat all individuals within one group
20 the same. Rather, the individualization model according to one embodiment of the present invention will account for individual-specific molar ratios corresponding to an individual's phenotype. This individual-specific information will be used to generate a dose specific for
25 each individual. In addition, the dosing model will not require a 24-hour N-acetylamonafide measurement.

In accordance with the present invention, an assay system will be provided that can be used in a clinical environment, whereby phenotypic determinants can be

quantified from a urine sample and applied to an individualization model to determine a dosage of amonafide for treating an individual which at least corresponds to the individual's ability to metabolize NAT2. As a result,
5 physicians will be provided with a tool for the individualization of therapy providing an alternative to the arbitrary selection of medications based on prognosis and categorical dosing.

In accordance with the present invention, an assay
10 system will be provided that can be used in a clinical environment, whereby phenotypic determinants can be quantified from a urine sample and applied to an individualization model to determine a dosage of amonafide for treating an individual that corresponds to the
15 individual's ability to metabolize NAT2. As a result, the physician will be provided with a tool for the individualization of therapy providing an alternative to the arbitrary selection of medications based on prognosis and categorical dosing.

20 While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the
25 principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features

hereinbefore set forth, and as follows in the scope of the appended claims.

2020-06-08

EXAMPLE III**Design of Clinical Trial with an
Individualized Treatment Regimen for Amonafide Therapy****5 CLINICAL STUDY PROTOCOL**

An open label phase 2, multicenter study of
amonafide as second line therapy in individuals who have
relapsed following prior cytotoxic chemotherapy regimens
10 containing anthracyclines or taxanes for metastatic breast
cancer

15 SYNOPSIS

Protocol Title: An open label phase 2, multicenter study of amonafide as
second line therapy in individuals who have relapsed following
prior cytotoxic chemotherapy regimens containing
anthracyclines or taxanes for metastatic breast cancer

Version Date: 1 Feb 2002

Sponsor: Xanthus Life Sciences Inc.

Project Phase: Phase 2

Study rationale: Amonafide is metabolized by N-acetylation to an active
metabolite, N-acetyl-amonafide. Interindividual differences in
N-acetylation can explain the variability in amonafide-induced
myelosuppression. In spite of phenotype-specific dosing, there
is still within-group variability in myelosuppression.

Objectives: Primary:
Determine the individual individual's acetylator phenotype and
assign an appropriate individualized dose of amonafide based
on the metabolic capacity;
Demonstrate the decreased toxicity of amonafide by the
assignment of the appropriate/individualized dose of

amonafile;

Define phenotype-specific dosing nomogram.

Secondary:

Determine the response rate of amonafile in individuals who have relapsed following prior cytotoxic chemotherapy regimens containing anthracyclines or taxanes for metastatic breast cancer;

Determine the duration of response and time to disease progression;

Collect survival data.

Study design:

This is an open label, phase 2, multicenter study of amonafile as second line treatment in individuals who have relapsed following prior cytotoxic chemotherapy regimens containing anthracyclines or taxanes.

See the flowchart(s) for the procedure to be performed at each visit.

Number of individuals: Approximately 80-100 individuals in the study (80 evaluable).

Total No. of centers: Approximately 15 centers

Diagnosis and main criteria for inclusion Individuals with metastatic (stage IV) breast cancer who have relapsed following prior cytotoxic chemotherapy regimens containing anthracyclines or taxanes. Individuals must be older than 18 years old, and acetylator phenotyping information must be obtained prior to dosing.

Test article(s): Amonafide (benzisoquinolinedione)

Duration of Individual participation and study

Individual:

Screening: up to 14 days

Treatment: up to 4.5 months (6 cycles)

Follow-up: 12 months from the date of enrolment

Telephone follow-up: until death

Study:

Enrollment: 6 months

Total study duration: 12 – 18 months

Concomitant treatment - Individuals already receiving biphosphonates to treat pain due to bone metastases prior to study entry may continue treatment during the study.

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- Growth Factors may be given only as treatment in case of severe neutropenia or in case of febrile neutropenia.
- Erythropoietin may be given in case of anemia.

Study Procedures

Screening (Day -14 to Day 0)

- The following assessments need to be completed within 14 days prior to treatment: demographic data, medical history, Karnofsky performance status, NYHA cardiac classification, complete physical exam, height and weight, vital signs, pregnancy test (women of childbearing potential), chest X-ray, ECG (12-lead), hematology, blood chemistry and urinalysis. A clinical assessment including adverse event review, concurrent medication and concurrent illness will also be done.

- The baseline tumor assessment will include a chest CT scan for all individuals, a bone scan for individuals who had an abnormal scan 3 months prior to study entry and an abdominal CT scan for individuals with abnormal liver chemistries or abdominal pain that had a past abnormal CT scan, ultrasound or MRI.

Phenotype evaluation: All eligible individuals will undergo acetylator (NAT2) and Cytochrome 450 1A2 (CYP1A2) phenotyping prior to and after the administration of amonafide. Caffeine will be used as a substrate to determine phenotyping. Caffeine (100 mg) will be administered as a tablet. Three hours after caffeine ingestion, the individuals will void their bladder and the urine will be discarded. A urine sample will be obtained 4-5 hours after caffeine ingestion.

Treatment Phase (up to 4.5 months)

- At each treatment cycle (21 day), amonafide will be infused daily for 5 days. During the dosing period and up to 1 hour after the end of the infusion, amonafide infusion-related adverse events will be assessed.

The following assessments will be completed every 3 weeks during treatment with amonafide: targeted physical exam, Karnofsky performance status, weight, vital signs, hematology, blood chemistry and urinalysis. A clinical assessment including adverse event review, concomitant medication and concurrent illness will also be done.

One week after the end of first treatment, individuals will be clinically evaluated (targeted physical exam, weight, vital signs, Karnofsky performance status, adverse event review, concomitant medication and concurrent illness) and the following laboratory evaluations will be performed:

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hematology, blood chemistry and urinalysis.

Phenotype evaluation will be performed (day 12).

Tumor assessment will be performed every 6 weeks (day 1 of cycles 2, 4, 6) during treatment with amonafide.

Quality of Life questionnaires EORTC QLQ-C30 and QLQ-BR23 will be undertaken prior to administration of amonafide at baseline and on day 1 of cycles 2, 4, 6.

End of Treatment

Individuals will be seen 1 week after the last treatment cycle with amonafide (Day 28) and the following tests will be completed: complete physical exam, Karnofsky performance status, weight, vital signs, Quality of Life questionnaires EORTC QLQ-C30 and QLQ-BR23, chest X-ray, hematology, blood chemistry, urinalysis, pregnancy test (women of childbearing potential) and ECG. A clinical assessment including adverse event review, concomitant medication and concurrent illness will also be done.

Study termination visit

Individuals discontinued from the study for any reason will be asked to come for a study termination visit. Parameters assessed will be: complete physical exam, Karnofsky performance status, weight, vital signs, Quality of Life questionnaires EORTC QLQ-C30 and QLQ-BR23, chest X-ray, hematology, blood chemistry, urinalysis, pregnancy test (women of childbearing potential) and ECG. A clinical assessment including adverse event review, concomitant medication and concurrent illness will also be done.

Follow-Up Visits

- After completion of study treatment or after early withdrawal from study treatment, individuals will be followed every 3 months for survival. Individuals will be assessed for 12 months from the date they were enrolled in the study or until death if it occurs earlier. Parameters assessed will be: complete physical exam, Karnofsky performance status, weight and vital signs. A clinical assessment including serious adverse event review will also be done.
- Following the end of the follow-up, individuals will be called every 3 months to assess survival.

Criteria for evaluation Efficacy

Primary: Overall response rate (partial and complete) and time to disease progression.

Secondary: Duration of response and survival.

Safety

All individuals who receive at least one dose of amonafide will be included in the safety analysis.

**Statistical method
General analysis plan
Rationale for number
of individuals**

To evaluate the overall response rate, two sets of analyses will be performed, one based on the intent-to-treat approach (all enrolled eligible individuals) and one based on data from evaluable individuals only, i.e. all eligible individuals who complete at least two cycles of therapy and undergo tumor evaluation. The analysis on time to disease progression will include all eligible individuals who received at least one treatment cycle.

All individuals who receive at least one treatment dose will be included in the safety analysis. Any incidence of adverse events will be recorded and classified according to body region and toxicity grade.

An interim analysis will be planned when 20-30 individuals will have been enrolled in the study and have completed at least 2 treatment cycles and underwent tumor evaluation.

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SCHEDULE OF EVENTS

TEST	STUDY PERIOD					
	Screening	Treatment Phase		End of Treatment	Follow-up	
	Day -14 to Day 0	Day 1 Cycle 1 Baseline	Day 12 Cycle 1	Day 28 of Final Cycle or Study Termination	Follow-up Visits (every 3 months)	Long-term follow-up (every 3 months)
Visit Number (Vt) / Week (Wk)	Vt 1 Day -14 to 0	Vt 2 Wk 1	Vt 3 Wk 2	Vt 9 Wk 19		
Informed Consent	X					
Complete medical history	X					
Complete physical exam	X	X		X	X	
Targeted physical exam			X			
Weight, height ⁽¹⁾	X	X	X	X	X	
Vital Signs (BP, pulse, temp.)	X	X	X	X	X	
Karnofsky performance status	X		X	X	X	
ECG (12 lead)	X			X		
NYHA classification	X					
Pregnancy test ⁽²⁾	X			X		
Hematology ⁽³⁾	X		X	X		
Blood chemistry ⁽⁴⁾	X		X	X		
Urinalysis	X		X	X		
Clinical assessment	X ⁽⁵⁾	X ⁽⁶⁾	X ⁽⁵⁾	X ⁽⁶⁾	X ⁽⁷⁾	
Tumor Assessment	X					
Phenotype evaluation	X		X			
QoL		X ⁽⁹⁾		X ⁽⁹⁾		
Chest X-Ray (PA and lateral)	X			X		
Survival					X	X
Test article administration		X ⁽¹⁰⁾				

(1) Height done at screening only.

(2) Women of childbearing potential only.

(3) Hemoglobin, Hematocrit, Leucocytes, Neutrophils, Basophils, Eosinophils, Lymphocytes, Monocytes, Erythrocytes and Platelets.

(4) Albumin, Alkaline phosphatase, Amylase, total Bilirubin, Bicarbonate, BUN or urea, Calcium, Chloride, Creatinine, Glucose, LDH, Phosphorus, Potassium, ALT, AST, Sodium, Total Triglycerides, Uric acid.

(5) Includes Adverse Event Review (AEs and SAEs), Concomitant medications, and Concurrent illness.

- (6) During the amonafide treatment (daily infusion for 5 days), infusion-related adverse events will be assessed.
- (7) Includes assessment of Serious Adverse Events only.
- (8) Tumor assessment to be done on the first day of cycle 2, 4, and 6 (Vts 4, 6, 8) only.
- (9) Quality of Life questionnaires EORTC QLQ-C30 and QLQ-BR23 will be done at baseline, on the first day of cycle 2, 4 and 6 (Vts 4, 6, 8) prior to the administration of amonafide and at the study termination visit (Vt 9).
- (10) For each treatment cycle, amonafide will be infused daily for 5 days.

INTRODUCTION

Metastatic Breast Cancer - Disease Background

5 Excluding skin cancers, breast cancer is the most
common cancer among women, accounting for nearly one of
every three cancers diagnosed in North American women. It
is also the second most common cause of cancer-related
10 deaths in women, after lung cancer. It is estimated that,
in 1999, breast cancer accounted for approximately 18,700
cases in Canada and approximately 175,000 cases in the
U.S. (Landis et al. 1999 CA Cancer J. Clin. 49(1): 8-31).
Breast cancer accounted for approximately 5,500 deaths in
Canada in 1999 and approximately 44,000 deaths in the U.S.
15 in 1997 (Canadian Cancer Statistics 2000, Toronto, Canada,
NCIC, Vol. 2000).

Even if significant advances have been made in the
treatment of early breast cancer, treatment options for
advanced breast cancer are still limited and the outcome
20 of metastatic (stage IV) breast cancer is almost always
fatal.

Women diagnosed with metastatic (stage IV) breast
cancer have a median survival expectancy of 17 to 20
months with a 5-year survival rate of only 15%. The 5-
25 year survival rate reaches 30-40% in women with bone
metastases only. Individuals with the poorer prognosis
are women with visceral disease, particularly liver
metastases: their median survival is of 8 to 10 months
only.

30 The primary treatment for metastatic breast cancer
consists of adjuvant systemic therapy using chemotherapy,

hormonal therapy or both. In individuals who received adjuvant chemotherapy with cytotoxic drugs as first-line therapy, such as 5-FU, doxorubicin, and cyclophosphamide (FAC), only 16.6% achieved complete remission as defined
5 by most clinical investigators (Hortobagyi 1998 N. Eng. J. Med. 339(14): 974-984). Furthermore, those who relapse after adjuvant therapy will have partial or complete drug resistance and be less amenable to a successful therapeutic intervention. Disease responsiveness to
10 chemotherapy declines in second-line and third-line therapies, in terms of response rate and time to disease progression.

New treatment strategies to address the development of drug resistance are then imperative to
15 increase the survival rate of individuals with metastatic breast cancer.

Amonafide Background

Amonafide (Benzisoquinolinedione, NSC 308847) is
20 an imide derivative of naphthalic acid (Brana et al. 1980 Cancer Chemother Pharmacol. 4: 61-66). Naphthalic acid is of interest because of its inhibitory effect on cellular replication. The cytotoxic activity of naphthalic acid is maximal when its side chain is composed of two methylene
25 groups with terminal nitrogen. Amonafide meets this requirement and has demonstrated significant activity against the L1210 and p388 leukemia cells lines as well as B16 melanoma and M5076 sarcoma cell line. Amonafide is a site-specific intercalating agent and a topoisomerase II

inhibitor (Warning *et al.* 1979 *Nucleic Acids Res.* 7: 217-230; Hsiang *et al.* 1989 *Mol. Pharmacol.* 36: 371-376).

Preclinical toxicity studies in mice, dogs, and rats indicate that amonafide produces reversible
5 hematopoietic, gastrointestinal, renal and hepatic toxicity. In addition, signs indicative of neurotoxicity were seen in dogs in both single-bolus and daily for 5 days schedules at the highest doses administered.

10 Clinical Pharmacology

N-acetylation is an important pathway for the metabolism of aromatic and hydrazine drugs (Weber and Hein (1985) *Pharmacol. Rev.* 37:25-79). Examples of commonly used drugs metabolized by N-acetylation include
15 procainamide, dapsone, aminogluthethimide, isoniazid, hydralazine, sulfasalazine and caffeine. The acetylator phenotype is inherited autosomally, with the allele for the slow phenotype (homozygous) predominating in the North American population. The estimate incidence of slow
20 acetylators is 60%, with the remaining 40% considered either rapid or indeterminate acetylators. The true "rapid" acetylator is a homozygote (5% of population) for the less common rapid allele, whereas the indeterminate phenotype (called rapid by some authors) is a
25 heterozygote, comprising approximately 35% of the population. There are significant ethnic and geographic correlates with acetylator phenotype (Weber and Hein, *supra*; Clark 1985 *Drugs* 29: 342-375). In particular, the slow acetylator phenotype is relatively uncommon (<10%) in

Oriental and Eskimo, whereas Mid-Eastern populations are comprised almost exclusively of slow acetylators.

Preliminary human pharmacology studies have reported that the volume of distribution of amonafide is large and that it is highly bound to body tissues. Amonafide is extensively metabolized and metabolites have been detected in both plasma and urine. Amonafide is metabolized by both N-acetylation and N-oxidation (Felder et al. 1987 Drug Metab. Dispos. 15: 773-778). It has been reported that N-acetyl-amonafide is approximately equipotent to the parent drug, whereas N-oxide-amonafide is essentially inactive (Felder et al. 1987 Drug Metab. Dispos. 15: 773-778). The degree of acetylation may account for the variability in pharmacokinetics and in the toxicity seen at the various doses tested, since N-acetyl-amonafide is cytotoxic (Felder et al. 1987 Drug Metab. Dispos. 15: 773-778).

Phase I studies were performed at the UTSA (University of Texas at San Antonio) (Saez et al. 1989 J. Clin. Oncol. 7: 1351-1358), OSU (Ohio State University) (Leiby et al. 1988 Proc. Am. Assoc. Cancer Res. 29: 278) and MDA (M. D. Anderson) (Legha et al. 1987 Cancer Treat. Rep. 71: 1165-1169). When given as a single bolus, the maximum tolerated dose (MTD) was 800 mg/m². The dose-limiting toxicity was myelosuppression (Saez et al. 1989 J. Clin. Oncol. 7: 1351-1358). When daily time five dose was given every 21 days, the MTD was 400 mg/m² ⁽¹²⁾ or 250 mg/m² (Leiby et al. 1988 Proc. Am. Assoc. Cancer Res. 29: 278). Again myelosuppression was the dose-limiting toxicity. Other reported toxicities were mild to moderate

nausea, vomiting and alopecia. All three centers reported acute toxicity with rapid amonafide infusion. This constituted of local inflammatory reactions, diaphoresis, flushing, tinnitus, headache and/or dizziness. Increasing
5 the duration of infusion to 1 hour minimized these effects. Regarding the different regimens, no schedule dependency was noted. The bolus dose MTD was different in two studies. The UTSA group (Saez et al. 1989 J. Clin. Oncol. 7: 1351-1358) showed a highly variable and
10 individualized hematotoxicity at sub-MTD doses, although they did reach a MTD of 800 mg/m². The OSU study (Leiby et al. 1988 Proc. Am. Assoc. Cancer Res. 29: 278) reported an MTD of 1125 mg/m² but noted considerable and variable toxicity. The same group also compared bolus dose (1125
15 mg/m²) to a 5-day schedule at 288 mg/m² and concluded that the 5-day schedule was preferred since more drug could be given (Leiby et al. 1988 Proc. Am. Assoc. Cancer Res. 29: 278). In another 5-day schedule, the MDA study (Legha et al. 1987 Cancer Treat. Rep. 71: 1165-1169) found a higher
20 MTD of 400 mg/m². The recommended Phase 2 doses were 200 mg/m² and 400 mg/m² (300-320 mg/m² in poor risk individuals).

From 1990 to 1998, 33 Phase 2 trials and 1 Phase 3 trial were conducted by different groups including the
25 Gynecologic Oncology Group (GOG), the South Western Oncology Group (SWOG), the Eastern Cooperative Oncology Group (ECOG), the Cancer and Leukemia Group B (CALBG), and the Illinois Cancer Center Trial. A total of 981 individuals received amonafide for a number of tumor

types. For most studies, the dose and schedule of amonafide was 300 mg/m² daily for 5 days every 3 weeks.

Three Phase 2 trials were conducted in metastatic breast cancer, as first line chemotherapy: 2 were done
5 with a dosage regimen of 800-900 mg/m² over 3 hours repeated every 4 weeks (Kornek et al. 1994 Eur. J. Cancer 30A(3): 398-400; Scheithauer et al. 1991 Breast Cancer Res. Treat. 20(1): 63-67)) and one with 300 mg/m² daily for 5 consecutive days every 3 weeks for 4 cycles,
10 followed by CAF (Costanza et al. 1995 Clin. Cancer Res. 1: 699-704). In the only Phase 3 trial, individuals received 300 mg/m² 4 times a day for 5 consecutive days every 3 weeks for 4 cycles, followed by CAF (Costanza et al. 1999 J. Clin. Oncol. 17(5):1397-1406). Amonafide was found to
15 be an active compound for the treatment of individuals with advanced breast cancer, with a response rate ranging from 18% to 50% in individuals with grade 3 or 4 thrombocytopenia.

NAT2 enzyme polymorphism seems to play an
20 important role in the metabolism of amonafide. In 1991, Ratain et al.(Ratain et al. 1991 Clin. Pharmacol. Ther 50(5): 573-579) investigated a phenotyping procedure for acetylation using caffeine as a probe substrate in individuals with cancer treated with amonafide. Slow and
25 fast acetylators of both caffeine and amonafide were identified. The production of the acetylated metabolite was one of the major determinants of myelosuppression, since fast acetylators had greater toxicity than slow acetylators (Ratain et al. 1991 Clin. Pharmacol. Ther 50(5): 573-579).
30 However, the area under the plasma

concentration-time curve of amonafide was significantly greater in fast acetylators, who would be expected to have a higher clearance and a lower area under the plasma concentration time-curve. This appeared to be an unusual finding compared to other drugs metabolized by N-acetylation. For most drugs, slow acetylators are at greater risk of adverse events. The unexpected behavior of amonafide was due to the inhibition of amonafide oxidation by N-acetyl-amonafide, since in vitro studies demonstrated that amonafide is a substrate for CYP1A2 and amonafide's oxidation is inhibited by its acetylated metabolite (Ratain et al. 1995 J. Clin. Oncol. 13: 741-747). Based on the acetylator phenotyping, new recommended doses were proposed for slow acetylators (375 mg/m² /day) and fast acetylators (250 mg/m² /day) (Ratain et al. 1993 Cancer Res. 53: 2304-2308). A fixed dose of amonafide of 300 mg/m² was considered inappropriate for all individuals, as fast phenotypes would be expected to experience grade 4 toxicity and slow phenotypes may be significantly under dosed (Ratain et al. 1991 Clin Pharmacol. Ther. 59(5): 573-579). Since there was still significant inter individual variability in toxicity at these new dose levels, a subsequent study attempted to develop pharmacodynamic models to individualize amonafide's dosing. The optimal model was defined by acetylator phenotype, pretreatment white blood cell count (WBC) and gender (Ratain et al. 1996 Pharmacogenetics 6: 93-101).

This protocol has been designed to assess safety and efficacy of amonafide as a second line treatment in

metastatic breast cancer women, assigned to individualized doses based on acetylator phenotype information and on pretreatment WBC.

5 **STUDY OBJECTIVES**

Primary

The primary objectives for this study are to:

- 10 1) determine the individuals acetylator phenotype and assign an appropriate individualized dose of amonafide based on metabolic capacity;
- 2) demonstrate the decreased toxicity of amonafide by the assignation of the appropriate/individualized dose or amonafide;
- 15 3) define a phenotype-specific dosing nomogram.

Secondary

The secondary objectives for this study are to:

- 20 1) determine the response rate of amonafide in individuals who have relapsed following prior cytotoxic chemotherapy regimens containing anthracyclines or taxanes for metastatic breast cancer;
- 25 2) determine the duration of the response and time to disease progression;
- 3) collect survival data.

SELECTION OF STUDY POPULATION

All individuals must take part in the consent process. During the consent process, the person obtaining consent must inform the individuals of all elements of informed consent. Adequate time must be allowed for questions and for the individuals to make voluntary decision. No protocol specific procedures will be performed before the individuals has signed and dated an EC approved informed consent document. The study begins with the signing and dating of the informed consent document. Individuals must also meet inclusion and exclusion criteria to be enrolled in the study.

INCLUSION CRITERIA

Individuals must fulfill all of the following criteria to be eligible for study selection:

Women of over 18 years;

- a) histologically confirmed metastatic (Stage IV) breast cancer;
- b) individuals who relapsed following prior cytotoxic chemotherapy regimens containing anthracyclines or taxanes (docetaxel-based or paclitaxel-based therapy);
- c) measurable and/or evaluable disease by radiographic means or physical examination;
- d) no other antitumor therapy in the previous months (6 months in case of prior anthracyclines exposure, 4 months in case of prior taxanes exposure);

- 20320-4662800F
- e) karnofsky score 70 (Table 25) with an expected survival of 12 weeks;
 - f) left Ventricular Ejection Fraction (LVEF) > 50%;
 - 5 g) adequate renal function as evidenced by the following laboratory tests:
 - h) serum creatinine < 1.5 X ULN;
 - i) adequate hepatic function as evidenced by the following laboratory tests:
 - 10 j) serum bilirubin < 1.5 X ULN;
 - k) alkaline phosphatase $\leq 2.5 \times \text{ULN}$ ($\leq 5 \times \text{ULN}$, in case of liver metastases);
 - l) serum AST or ALT $\leq 2.5 \times \text{ULN}$ ($\leq 5 \times \text{ULN}$, in case of liver metastases);
 - 15 m) adequate hematologic status as evidenced by the following laboratory tests:
 - n) total WBC $\geq 3.0 \times 10^9/\text{L}$ or 3,000/mm³;
 - o) neutrophils $\geq 1.5 \times 10^9/\text{L}$ or 1,500/mm³;
 - p) platelets $\geq 100 \times 10^9/\text{L}$ or 100,000/mm³;
 - 20 q) hemoglobin $\geq 10.0 \text{ g/dL}$ or 10g/100 mL;
 - r) no blood transfusion within the previous 2 weeks of signature of the informed consent;
 - s) negative pregnancy test (urine or blood) and an effective contraception method in women of childbearing potential. Reliable contraception must have been started 4 weeks prior to signature of the consent and must be continued throughout the study and until at least four weeks after completion of study treatment. A
 - 25

woman of childbearing is defined as one who is biologically capable of becoming pregnant. This includes women who are using contraceptives or whose sexual partners are either sterile or using contraceptives;

t) previous radiotherapy is allowed if the end of the treatment was more than 30 days prior to signature of the informed consent;

u) no underlying illness likely in the view of the investigator to be a danger to being enrolled in the trial;

v) expected co-operation of the individual for the treatment and follow-up must be obtained and documented;

w) phenotyping procedures must have been completed successfully; and

x) written informed consent must be obtained and documented.

Table 25

Karnofsky Performance Scale

Scale	Description
100	Normal, no complaints, no evidence of disease
90	Able to carry on normal activity, minor symptoms or signs of disease
80	Normal activity with effort, some signs or symptoms of disease
70	Cares for self, unable to carry on normal activity or to do active work

60	Requires occasional assistance, but is able to care for most of own needs
50	Requires considerable assistance and frequent medical care
40	Disabled, requires special care and assistance
30	Severely disabled, hospitalization is indicated although death is not imminent
20	Hospitalization necessary, very sick, active supportive treatment necessary
10	Moribund, fatal processes progressing rapidly

Source: Schag CC, Heindrich RL, Ganz PA: Karnofsky Performance Status Revised: Reliability, Validity and Guidelines. Clinical Oncology 2: 187-193, 1984.

EXCLUSION CRITERIA

Individuals must not meet any of the following criteria to be eligible for study selection:

- a) myocardial infarction within 3 months of signature of the informed consent;
- b) unstable angina pectoris, cardiac insufficiency (NYHA Class III-IV; see Table 26), uncontrolled arrhythmia, or uncontrolled hypertension at the time of signature of the consent form;
- c) clinically significant abnormal hematological parameters other than those defined in the inclusion criteria;

- 5
- d) clinically significant abnormal biochemical parameters other than those defined in the inclusion criteria;
 - e) history or presence of brain or leptomeningeal metastases;
 - f) ascites, pleural effusions, or osteoblastic bone metastases as the only site of measurable and evaluable disease;
 - 10 g) prior major surgery less than 4 weeks before signature of the informed consent. Individuals must have recovered from prior surgery;
 - 15 h) individuals who received treatment with Growth Factors (i.e. G-CSF, GM-CSF) within 2 weeks of the signature of the informed consent;
 - i) hormonal anticancer therapy within 30 days of the signature of the informed consent;
 - j) pregnant or lactating women;
 - k) clinically significant active infections;
 - 20 l) other prior malignancies, except for cured non melanoma skin cancer or curatively treated *in situ* carcinoma of the cervix;
 - m) other serious illness or medical condition;
 - 25 n) individuals with a history of a psychological illness or condition which may interfere with the individuals ability to understand the requirements of the study;
 - o) individuals who received an investigational new drug within the last 30 days;

- p) pre-existing neuropathy (motor or sensor) greater than grade 2;
- q) any other condition which in the investigator's opinion would not make the individuals a good candidate for the trial.

5

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Table 26
New York Heart Association (NYHA) Functional Classification

Class	Description
Class I	Individuals with cardiac disease but without limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain.
Class II	Individuals with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain.
Class III	Individuals with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnea, or anginal pain.
Class IV	Individuals with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of cardiac insufficiency or of the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased.

5

Source: Oxford Textbook of Medecine, Vol 2, pp. 2228. Oxford University Press. 1997.

Number of individuals

10

Approximately 15 centers will participate in this study to be conducted in Europe and in North America. Chosen centers will have specialized expertise in oncology and experience in the conduct of clinical trials.

Each site will enroll a maximum of 6 individuals for a total individual population of 80-100.

Screening Failures

5 Individuals who fail to meet the inclusion and/or exclusion criteria are defined as screen failures. Individuals that will undergo phenotyping procedures but will never receive the test article will be considered screening failures. The investigator is to maintain a
10 screening log for all screen failures that documents the individual screening number, individual initials, and reason(s) for screen failure. A copy of the log should be retained in the investigator's study files.

15 **PRIOR TREATMENT**

Reasonable efforts will be made to determine all relevant treatment received by the individual within 30 days before administration of the test article. All relevant information must be recorded on the individual's
20 CRF. Include the name of the procedure or drug and other information required on the CRF.

CONCOMITANT TREATMENT

25 **Permitted Treatment**

Individuals already receiving biphosphonates to treat pain due to bone metastases prior to signature of the informed consent may still continue biphosphonate treatment during the study.

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Growth Factors (including G-CSF, and GM-CSF) may be given only as treatment in case of severe neutropenia (neutrophils $< 500 \times 10^9/L$ or $500/mm^3$) lasting more than 5 days or in case of febrile neutropenia.

- 5 Hematinics (erythropoietin) may also be given in case of anemia. Blood transfusions (platelets, packed cells) can also be given.

Prohibited Treatment

- 10 Treatment with hormonal anticancer therapy (tamoxifen, raloxifen or toremifene) is not allowed during the study. Hormone replacement therapy is allowed if it was started prior to enrolment in the study.

- 15 Other systemic anticancer agents including agents who modulate the immune response to cancer and immunosuppressive agents are prohibited.

- 20 Concurrent systemic steroids use is prohibited. Topical and inhaled steroids are allowed if the individuals were already taking them for dermatological conditions or for allergy/asthma prevention.

- 25 Radiation therapy directed at the treatment of indicator lesions during the study treatment period will not be allowed. Individuals who have received radiotherapy at these indicator lesions will be withdrawn from the study.

Treatment with other investigational agents is not allowed during the study.

TRIAL DESIGN

Description

This is an open label, phase 2, multicenter study of amonafide as second line treatment in individuals with metastatic breast cancer who have relapsed following prior
5 cytotoxic chemotherapy regimens containing anthracyclines or taxanes.

The total duration of this study will be of 12-18 months: 6 months for enrollment, maximum of 5 months for treatment and up to 7 months for individual follow-up
10 after completion of treatment with amonafide.

Procedures

See the schedule of events - flowchart for the procedures to be performed at each visit.
15

Screening (Visit 1, Day -14 to Day 0)

The following assessments need to be completed within 14 days prior to treatment:

- 1) Signed written informed consent form;
- 20 2) Collection of demographic data;
- 3) Complete medical history including dates and description of initial diagnosis and documentation of any cancer therapies and results of the prior assessment of the primary
25 tumor overexpression to HER-2/neu receptors (if available);
- 4) Complete physical exam;
- 5) Height and weight,
- 6) Vital signs (pulse, blood pressure, oral or
30 tympanic temperature);

- 7) Karnofsky performance status (see Table 25);
8) New York Heart Association Classification (NYHA class, see Table 26);
9) Tumor assessment;
5 10) Pregnancy test (women of childbearing potential only);
11) Chest X-ray (anteroposterior [AP] and lateral);
12) ECG (12-lead);
10 13) Phenotyping procedures (see section 15.1)
14) Hematology;
15) Blood chemistry;
16) Urinalysis;
15 17) Clinical assessment (adverse event, concomitant medication, concurrent illness).

The results of all tests will be entered in the CRF provided.

20 **Tumor assessment**

At baseline, tumor lesions will be categorized as measurable or non-measurable:

25 Measurable is defined as any lesion that can be accurately measured in at least one dimension (longest diameter to be recorded) as 20 mm with conventional techniques (physical examination, X-ray, computerized tomography (CAT), magnetic resonance imaging (MRI) or ultrasound) or as 10 mm with spiral CT scan.

Non-measurable is defined as all other lesions, including small lesion (longest diameter < 20 mm with conventional techniques or < 10 mm with spiral CT scan) and truly non-measurable lesions (i.e. bone lesions, 5 leptomeningeal disease, ascites, pleural/pericardial effusion, inflammatory breast disease, lymphangitis cutis/pulmonis, abdominal masses that are not confirmed/followed by imaging techniques and cystic lesions) .

10

All measurements should be recorded in metric notation, using a ruler or calipers. The technique used will be left to the discretion of the investigator, however for each individual, the same technique must be 15 used throughout the study. The measurements should be made by the same investigator for all assessments for each individual.

It is essential that target lesions be measured by the same method throughout the study so that comparison is 20 consistent. It is also essential that target lesions documented at the beginning of the study be continuously evaluated throughout the study. Clinically detected lesions must first be evaluated not more than 2 weeks prior to the first administration of amonafide. CT scans, 25 X-ray and MRI scans must be done within 2 weeks prior to the administration of amonafide.

The baseline tumor assessment will include a chest CT scan for all individuals, a bone scan for individuals who had an abnormal scan 3 months prior to study entry and 30 an abdominal CT scan for individuals with abnormal liver

2003-09-09 10:00:00

chemistries or abdominal pain that had a past abnormal CT scan, ultrasound or MRI.

All measurable lesions up to a maximum of 10 lesions representative of all organs should be identified as target lesions and for any one tissue or organ, up to maximum of 5 lesions should be selected. All lesions will be recorded and measured at baseline and ideally, all target lesions should be measured at each assessment. Target lesions should be selected on the basis of their size (lesions with the longest diameter(s)) and their suitability for accurate repetitive measurements.

Unidimensional target lesions:

A sum of the longest diameter for all target lesions will be calculated and reported as the baseline of sum of the longest diameter. The baseline sum of the longest diameter will be used as reference to further characterize the objective tumor response of the measurable dimension of the disease.

20

Bidimensional target lesions:

A sum of the products of the longest diameter by the greatest perpendicular diameter for all target lesions will be calculated and reported as the baseline sum of the products. The baseline sum of the products will be used as reference to further characterize the objective tumor response of the measurable dimensions of the disease.

30

Non-target lesions:

All other lesions (or sites of disease) should be identified as non-target lesions and should also be recorded at baseline. Measurements are not required and
5 these lesions should be followed as "present" or "absent".

Baseline visit (Visit 2; Day 1 of cycle 1)

Day 1 of cycle 1 is the first day of amonafide
10 infusion. Prior to the administration of the test article, the following assessments must be performed:

- 1) Quality of Life questionnaires EORTC QLQ-C30 and QLQ-BR23 (see Table 27);
- 15 2) Complete physical exam;
- 3) Weight;
- 4) Vital signs (pulse, blood pressure, oral or tympanic temperature); and
- 20 5) Clinical assessment (adverse event, concomitant medication, concurrent illness).

Table 27
EORTC Quality of Life Questionnaire



EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials: d d d d
 Your birthdate (Day, Month, Year): 9 9 9 9 9 9
 Today's date (Day, Month, Year): 9 9 9 9 9 9

	Not at all	A little	Quite a bit	Very much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a <u>long</u> walk?	1	2	3	4
3. Do you have any trouble taking a <u>short</u> walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4
During the past week:				
	Not at All	A little	Quite a bit	Very much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

2003-09-09 10:00:00

For the following questions please circle the number between 1 and 7 that best applies to you

1 2 3 4 5 6 7
Very poor Excellent

1 2 3 4 5 6 7
Very poor Excellent



EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials: d d d d
 Your birthdate (Day, Month, Year): 9 9 9 9 9 9
 Today's date (Day, Month, Year): 9 9 9 9 9 9

	Not at all	A little	Quite a bit	Very much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a <u>long</u> walk?	1	2	3	4
3. Do you have any trouble taking a <u>short</u> walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:

	Not at All	A little	Quite a bit	Very much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

200320-962801

During the past week:	Not at all	A little	Quite a bit	Very much
17. Did you have any pain in your arm or shoulder?	1	2	3	4
18. Did you have a swollen arm or hand?	1	2	3	4
19. Was it difficult to raise your arm or to move it sideways?	1	2	3	4
20. Have you had any pain in the area of your affected breast?	1	2	3	4
21. Was the area of your affected breast swollen?	1	2	3	4
22. Was the area of your affected breast oversensitive?	1	2	3	4
23. Have you had skin problems on or in the area of your affected breast (e.g. itchy, dry, flaky)?	1	2	3	4

At each treatment cycle, amonafide will be infused daily for 5 days. During the dosing period and up to 1 hour after the end of the infusion, amonafide infusion-related adverse events will be assessed.

5 A treatment cycle is defined as the period of time starting with the first dose of amonafide (Day 1 of the cycle) and ending 21 day after this first dose. Neutrophils must be $> 1.5 \times 10^9/L$ ($1,500/mm^3$) and platelets $> 100 \times 10^9/L$ ($100,000/mm^3$) prior to the beginning of the
10 subsequent cycles.

Cycle 1 - Day 12 (Visit 3)

One week after the end of first treatment, individuals will be clinically evaluated (targeted
15 physical exam, weight, vital signs, Karnofsky performance status, adverse event review, concomitant medication and concurrent illness) and the following laboratory evaluations will be conducted: hematology, blood chemistry and urinalysis. Phenotyping procedures will also be
20 performed at this visit.

This visit may be advanced or delayed by only 1 day in relation with the visit timetable.

Treatment cycle (Visits 4, 5, 6, 7, 8; Day 1 of cycles 2, 3, 4, 5, 6)
25

The following parameters will be assessed every cycle (21 days) during treatment with amonafide. All tests should be performed prior to the administration of amonafide and each visit may be advanced or delayed by 2
30 days in relation with the visit timetable.

- 1) Physical exam
- 2) Weight;
- 3) Karnofsky performance status (see Table 25);
- 5 4) Vital signs (pulse, blood pressure, oral or tympanic temperature);
- 5) Hematology;
- 6) Blood chemistry;
- 7) Urinalysis.

10

A clinical assessment will also be completed, including:

- 1) Adverse event review;
- 2) Concomitant medication;
- 15 3) Concurrent illness.

At each treatment cycle, amonafide will be infused daily for 5 days. During the infusion period and up to 1 hour after the end of the infusion, amonafide infusion-related adverse events will be assessed.

20

Tumor assessment

Appropriate radiographic testing (chest X-rays, CT scans, or MRI scans) will be done to assess the tumor response. Tumor assessment will be performed every 6 weeks (day 1 of cycles 2, 4, 6) during treatment with amonafide.

Tumor response will be assessed in individuals to define complete response (CR), partial response (PR), stable disease (SD) and disease progression (PD) according

30

to the Response Evaluation Criteria In Solid Tumors (RECIST criteria) as defined in Table 28 In the case of differences in response category between assessments made by the CT-scan and X-ray, the CT-scan will be considered
5 the more accurate technique.

2020-06-23

Table 28
Measurability of Disease and Criteria for Analysis

Evaluation of target lesions (unidimensional)

Complete Response (CR)	Disappearance of all target lesions.
Partial Response (PR)	At least 30% decrease in the sum of the longest diameter of target lesions, taking as reference the baseline sum of the longest diameter.
Stable Disease (SD)	Neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum of the longest diameter since the treatment started.
Progressive Disease (PD)	Must meet one of the following criteria: At least a 20% increase in the sum of the longest diameter of target lesions, taking as reference the smallest sum of the longest diameter recorded since treatment started or; The appearance of one or more new lesions.

5

Evaluation of target lesions (bidimensional)

Complete Response (CR)	The disappearance of all known disease, determined by two observations not less than four weeks apart.
Partial Response (PR)	A 50% decrease in the sum of the products of the longest diameter by the greatest perpendicular diameter of all measurable lesions as determined by 2 observations not less than 4 weeks apart. No appearance of new lesions and no progression of any lesions.
Stable Disease (SD)	A 50% decrease in total tumor size cannot be established nor has a 25% increase in the sum of the products of the longest diameter by the greatest perpendicular diameter of all measurable lesions been demonstrated.
Progressive Disease (PD)	A 25% or more increase in the size of at least one bidimensionally measurable lesion or appearance of a new lesion.

Evaluation of non-target lesions

10

Complete Response (CR)	Disappearance of all non-target lesions for at least 4 weeks and normalization of tumor marker level.
------------------------	---

Non-Complete (Non-CR)	Persistence of one or more non-target lesions.
Non Progressive Disease (Non-PD)	Maintenance of tumor marker level above the normal limits.
Progressive Disease (PD)	Appearance of one or more new lesions or unequivocal progression of existing non-target lesions.

Evaluation of best overall response

5 The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurement recorded since the treatment started).

10

Target lesions	Non-target lesions	New lesions	Overall response
CR	CR	No	CR
CR	Non-CR/Non-PD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Source: Therass P, Arbuck SG, Eisenhauer EA, Wanders J, et al: New Guidelines to Evaluate the Response to Treatment in Solid Tumors. J Natl Cancer Inst 15 92(3):205-216, 2000.

The development of brain metastases will be considered as a sign of progression even if the disease is responding outside the brain. However, the investigator

may choose to continue the test article treatment if the individual is responding elsewhere.

It is assumed that the requirement for irradiation is indicative of progressive disease. In the case that irradiation has been given to any measurable lesions during the study, the individual will be assessed as having progressive disease from the date of irradiation. Individuals who have received radiotherapy at these indicator lesions will be withdrawn from the study.

Quality of Life questionnaires

EORTC QLQ-C30 and QLC-BR23 questionnaires will be performed every 6 weeks (day 1 of cycles 2, 4, 6) during treatment with amonafide. These questionnaires should be completed at the start of the visit prior to any treatment being given or any examinations/procedures (including the assessment for adverse events) are performed.

The results of all tests will be entered in the CRF provided.

End of Treatment or Early Termination Visit (Visit 9)

All individuals will be seen one week after their last treatment cycle (Day 28) with amonafide and the following tests will be completed:

- 1) Quality of Life questionnaires QLQ-C30 and QLQ-BR23 (see Table 27);
- 2) Completed physical exam;
- 3) Weight;
- 4) Karnofsky performance status (see Table 25);

- 4) Vital signs (pulse, blood pressure, oral or tympanic temperature);
- 5) Chest X-ray (anteroposterior [AP] and lateral);
- 5 6) Pregnancy test (women of childbearing potential);
- 7) Hematology;
- 8) Blood chemistry;
- 9) Urinalysis;
- 10 10) ECG (12-lead).

A clinical assessment will also be completed, including:

- 1) Adverse event review;
- 15 2) Concomitant medication;
- 3) Concurrent illness.

Follow-up visits

After completion of study treatment with amonafide or after early withdrawal from study treatment with amonafide, individuals will be followed every 3 months for up to 12 months from the date they were enrolled or until death if it occurs earlier.

- 25 1) Parameters assessed will be:
- 2) Complete physical exam;
- 3) Weight;
- 4) Karnofsky performance status (see Table 25);
- 5) Vital signs (pulse, blood pressure, oral or tympanic temperature).
- 30

A clinical assessment will also be completed, including:

- 1) Adverse event review;

5

Long-term follow-up

Individuals will be contacted every 3 months by telephone to assess survival.

10

Removal of Individuals from Test Article Administration or Assessment

Follow-up information will be obtained for individuals who discontinued or were withdrawn. See the schedule of events for procedures to be performed at final and follow-up visits.

15

The investigator will make every reasonable effort to keep each individual in the study. However, if the investigator removes a individual from the study or if the individual declines further participation, prior to any therapeutic intervention, final assessments will be performed if possible. All evaluations and observations, together with the description of the reason(s) for study withdrawal must be recorded in source documents and reported in the CRF.

20

25

Individuals who are removed from the study due to an adverse experience (clinical or laboratory) will be treated and followed according to accepted medical practice. All pertinent information concerning the

outcome of such treatment must be recorded in source documents and reported in the CRF.

The following are justifiable reasons for the investigator to withdraw an individual from the study:

5

1) Progressive disease. Any individual not achieving subjective benefit or at least stable disease after 2 treatment cycles will be removed from the study;

10

2) Unacceptable toxicity;

3) Withdrawal of consent: Where an individual has been in the study < 21 days, an additional individual will be recruited to replace him/her;

15

4) Unforeseen events: Any event which in the judgment of the investigator makes further treatment inadvisable;

5) Withdrawal by the physician for clinical reasons not related to the test article treatment;

20

6) Serious adverse event requiring discontinuation of test article treatment;

7) Serious violation of the study trial (including persistent individual attendance failure and non-compliance).

25

An effort must be made to determine why an individual fails to return for the necessary visits or is dropped from the study. Regardless of the reason for termination, all data available for the individual at the

30

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time of discontinuation of follow-up should be recorded in source documents and reported in the CRF. All reasons for discontinuation of treatment should be documented.

5 **LABORATORY DETERMINATIONS**

Only one laboratory will be used by each investigator for all determinations unless a special test is required. In such cases, an additional laboratory may be designated by the investigator for the special test only. A central laboratory will be used for the phenotyping evaluations. Details on handling of samples and sampling procedures are provided below.

Collection, Preparation, Labeling and Shipping of Samples

15 Collection/Preparation of sample for phenotyping evaluation:

Individuals will void their bladder 3 hours after caffeine ingestion and they will discard the urine. A urine sample will be obtained at 4-5 hours after ingestion.

The sample will be transferred immediately in six (6) polypropylene tubes of 5 mL and frozen in an upright position at -20°C until shipped to the central laboratory. Each tube will be labeled with already pre-printed labels.

Labeling

Samples will be identified with a label incorporating the assay(s) to be performed, protocol number, investigator name, individual number, and protocol

day and time of collection. Computer-printed labels with bar code will be provided by Xanthus Life Sciences Inc. If it should be necessary to hand write the information, only indelible ink should be used.

- 5 Preprinted labels will be applied to tubes at room temperature at least 2 hours before refrigerating or freezing. Adequate adhesion cannot be guaranteed if the labels are applied to cold tubes.

- 10 Labels will be applied so that the bar code can be scanned along the length of the tube. The bar code cannot be read if it wrapped circumferentially around the tube. Labels will not be spiralled or wrapped around the tube, as this also interferes with reading the bar code.

- 15 All samples must be kept frozen after collection and until shipment is initiated.

Sample shipment instructions

Mail only Monday through Wednesday via next day delivery courier service.

- 20 Fill a polystyrene container with dry ice.

Place the frozen samples in a plastic bag, and label the bag with the master protocol/site/individual identification label provided.

- 25 Place the bag in the container and cover completely with dry ice.

Enclose the white copy of the CRF page

Fax a copy of the paperwork with the courier information (including waybill number) to:

René Paulussen Ph.D. / Yves Blais Ph.D.

- 30 Xanthus Life Sciences Inc.

Fax: 514-843-9941

Send the frozen urine samples packed in dry ice
via 24 hour overnight courier to:

René Paulussen Ph.D. / Yves Blais Ph.D.

5 Xanthus Life Sciences Inc.

225 President Kennedy

Suite PK-5660

Montreal, Quebec H2X 3Y8

Canada

10

For any problems or questions please contact
Xanthus Life Sciences in Montreal:

	phone	e-mail
15 René Paulussen Ph.D.:	514-843-5959, ext 115	rene.paulussen@xanthusls.com
Yves Blais Ph.D.	514-843-5959, ext 109	yves.blais@xanthusls.com
or by fax:	514-843-9941	

Safety Laboratory Determinations

20 The following laboratory tests will be performed
at screening, Day 12 of cycle 1, Day 1 of cycles 2, 3, 4,
5, 6 and end of treatment.

25 1) Hematology: Complete blood count, including
platelets and white blood cells count with
differential.

30 2) Blood Chemistry: albumin, alkaline
phosphatase, amylase, total bilirubin,
bicarbonate, BUN or urea, calcium, chloride,
cholesterol, creatinine, glucose, LDH,

phosphorus, potassium, ALT, AST, sodium, total triglycerides, uric acid.

3) Urinalysis: pH, protein, blood, glucose.

5

DETERMINATION OF CYP1A2 AND NAT-2 PHENOTYPES

Analytical procedures

10 CYP1A2 phenotyping

The CYP1A2 phenotype is determined by measurement of the concentration ratio:

$$15 \quad \frac{1,7\text{-dimethylxanthine} + 1,7\text{-dimethyl uric acid}}{\text{caffeine}}$$

The concentration of caffeine and the sum of the concentration of the caffeine metabolites 1,7-dimethylxanthine and 1,7-dimethyl uric acid are determined by validated enzyme immunoassays.

20

Caffeine enzyme immunoassay

A specific antibody was raised in rabbits against caffeine conjugated to Keyhole Limpet Hemocyanin (KLH) through a spacer arm. The antibodies are immobilized on a microtiter plate. An aliquot of the sample is allowed to compete with a fixed amount of caffeine-horseradish peroxidase (HRP) conjugate for the limiting number of binding sites on the antibodies. After washing the plate a colorless substrate is added which is transformed to a

colored product proportionally to the amount of HRP bound. After stopping the reaction the color (absorbance) is measured.

The caffeine concentration in the samples is assessed from a caffeine standard curve included in the assay.

The caffeine enzyme immunoassay has previously been validated at Xanthus Life Sciences Inc.

10 *1,7-Dimethylxanthine and 1,7-dimethyl uric acid enzyme immunoassay*

For the measurement of the concentrations of 1,7-dimethylxanthine and 1,7-dimethyl uric acid an antibody was developed in rabbits that selectively binds both molecules from a urine sample. The assay is performed as described above for caffeine, employing a HRP-conjugate and standard curve specific for 1,7-dimethylxanthine and 1,7-dimethyl uric acid.

The 1,7-dimethylxanthine and 1,7-dimethyl uric acid enzyme immunoassay has previously been validated at Xanthus Life Sciences Inc.

NAT2 phenotyping

Caffeine will also be used to determine the N-acetyltransferase-2 (NAT2) activity, by measurement of the ratio:

1-methylxanthine (1X)

5-acetamino-6-amino-3-methyluracil (AAMU)

Measurements of 1X and AAMU will be performed by validated enzyme immunoassays.

1-Methylxanthine enzyme immunoassay

5 The concentration of 1X is measured using a specific rabbit antibody raised against a 1X derivative conjugated to KLH as carrier protein. The antibodies are immobilized on a microtiter plate. Following competition between a fixed amount of 1X-HRP conjugate and the 1X in
10 the sample (or standard) for the limiting number of immobilized antibody-binding sites, unbound material is washed away. Substrate is added and transformed to a colored product proportionally to the amount of HRP bound. After stopping the reaction, the color (absorbance) is
15 measured. The 1X concentration in the samples is determined from a 1X standard curve included in the assay.

The 1-methylxanthine enzyme immunoassay has previously been validated at Xanthus Life Sciences Inc.

20 *5-Acetamino-6-amino-3-methyluracil enzyme immunoassay*

Measurement of the AAMU concentration in urine samples is performed by a competitive enzyme immunoassay similar to the one described above for 1X. A specific antibody is immobilized on a microtiter plate, and the
25 AAMU-HRP conjugate and AAMU from the sample compete for the binding sites on the antibodies. After substrate incubation the color is measured and the AAMU concentration in the urine samples is determined from the standard curve included in the assay.

The AAMU enzyme immunoassay has previously been validated at Xanthus Life Sciences Inc.

Phenotyping Evaluation

5 Phenotyping evaluations will be done at screening and on Day 12 of cycle 1. Individuals will undergo phenotyping of N-acetyltransferase-2 (NAT2) and cytochrome P450 1A2 (CYP1A2) activity using caffeine. Caffeine will be administered as a tablet (100 mg), taken with 1 glass
10 of water.

 Individuals will void their bladder 3 hours after caffeine ingestion and they will discard the urine. A urine sample will be obtained at 4-5 hours after ingestion. The sample will be transferred immediately in
15 six (6) polypropylene tubes of 5 mL and frozen in an upright position at -20°C until shipped to the central laboratory. Each tube will be labeled with already pre-printed labels.

 Please refer to Collection, Preparation, Labeling
20 and Shipping of Samples, for additional details.

 At baseline, the investigator will be informed of the individual's acetylator phenotype by FAX within 5 working days following submission of the individual's urine sample.

25

TEST ARTICLE

20030920-96628001

Test Article Administration

Amonafide (the test article) is to be administered only to individuals who have provided informed consent and have been phenotyped.

5 All data concerning the test article administration will be recorded on source documents and will be checked during monitoring visits.

Following reconstitution and dilution, amonafide will be given over three (3) hours daily for 5 days. The
10 schedule of drug administration should be given every 21 days in the absence of disease progression or unacceptable toxicity for a maximum of 6 cycles. Infusion will be given through an established intravenous line. Amonafide is very unstable in dextrose-containing solutions and
15 these should not be used for administration.

Individuals must remain under medical supervision for 1 hour following completion of the infusion to assess infusion/post-infusion adverse events.

All doses of amonafide will be calculated on the
20 basis of milligrams of drug per square meter of body surface area as measured at baseline (mg/m^2). A nomogram for the assessment of body surface area from the individual height and weight is given in Fig. 29.

Initial amonafide dose will be determined by the
25 individual acetylator phenotype and pretreatment WBC. Based on the acetylator phenotyping, the individuals will be categorized as either slow, indeterminate/slow, indeterminate/fast, or fast acetylators. The individual will be further categorized as low or high pretreatment
30 WBC count (values ≤ 8 or $8 \times 10^9/\text{L}$ ($8000/\text{mm}^3$)).

Based on results from previous clinical studies, significant neutropenia can be anticipated with nadir counts on days 12-15 and recovery by approximately day 21. Thrombocytopenia may also occur. Neutrophils must be $> 1.5 \times 10^9/L$ ($1,500/mm^3$) and platelets $> 100 \times 10^9/L$ ($100,000/mm^3$) prior to the beginning of the subsequent cycles.

If the individual develops grade 4 myelosuppression following the initial cycle of treatment (nadir neutrophils $< 0.5 \times 10^9/L$ ($500/mm^3$) and/or platelets $< 25 \times 10^9/L$ ($25,000/mm^3$)), the daily dose of amonafide for the second cycle should be reduced by 25% or by $50mg/m^2$.

If the myelosuppression is grade 0 or 1 toxicity after the initial cycle of treatment (nadir neutrophils $> 2 \times 10^9/L$ ($2,000/mm^3$) and platelets $> 150 \times 10^9/L$ ($150,000/mm^3$)), the daily dose of amonafide for the second cycle should be increased by 20-25%.

Formulation, Packaging, and Labeling

Test article will be supplied in 5 mL single use vials containing 100 mg of drug as yellow to orange or red-orange lyophilized powder. The label will include the following information (as applicable): test article identification, lot number and expiry date, storage condition, warning statements, protocol number, section to write investigator name, section to write individual identification.

Storage and Stability

Intact vials should be stored under refrigeration (2-8°C). Solution reconstituted in normal saline exhibit no decomposition over 14 days either at room temperature or under refrigeration. However, the reconstituted solution contains no antibacterial preservatives and should be used immediately. It is advised that the reconstituted product be discarded 8 hours after initial entry.

Preparation

Appropriate aseptic technique should be used for test article reconstitution/preparation. Each vial should be reconstituted with 4 mL of sterile water for injection USP or of 0.9% Sodium Chloride Injection USP for an amonafide concentration of 25 mg/mL with a pH of 5 to 7.

Add the appropriate dose of amonafide to a bag of 100 mL of normal saline prior to administration. Amonafide is very unstable in dextrose-containing solutions and these should not be used for reconstitution or for administration.

Drug Accountability

Test articles for this trial are to be used only in accordance with this protocol and under the supervision of the investigators. The investigators will undertake not to destroy any labels or unused drug unless otherwise directed by Xanthus Life Sciences (XLS) Inc.

Regulatory agencies require accounting for the disposition of all investigational test articles received

by each clinical site. Information on test article disposition required by law consists of the date received, date administered, quantity administered, and the individual to whom the test article was administered. The investigator is responsible for the accounting of all unused test articles and all used test article containers. The investigator will use this information to maintain an accurate and complete dispensing and inventory record.

Supplies will be shipped to the investigative site at appropriate intervals, depending on individual accrual. XLS Inc. will provide a dispensing and inventory record for this trial. This form must be maintained either in the pharmacy or in another area approved by the sponsor monitor.

Each time a dose is prepared for an individual, the following information should be recorded: the individual's initial, the individual's study number, the total dose prepared, the number of vials used, the number of the lot from which the dose was prepared, and the initials of the person preparing the dose. Drug accounting will be reviewed by the monitor during routine monitoring visits. The monitor will ensure that the correct procedures are being followed and will satisfy him/her that the supplies are being adequately controlled and the inventory is being properly maintained and that storage conditions are in accordance with the label requirements.

At the termination of the study, a final drug accountability review and reconciliation must be completed and any discrepancies must be investigated and their

resolution documented. All used/unused test articles must be returned to the sponsor/the contract distribution center with the appropriate form.

5 MEASURES TO MINIMIZE/AVOID BIAS

Individual Identification

Individuals will be numbered in a manner specified by the sponsor. Individuals should be identified to the sponsor only by their assigned number, initials, date of birth, and sex. The investigator must maintain a list of individual names and the identifying information indicated above.

15 SAFETY

Safety Variables

Physical examinations, as well as vital signs and laboratory evaluations will be performed throughout the study.

Safety Assessment methods

Following the first treatment cycle of amonafide, each individual will be assessed for signs of adverse events and disease related signs and symptoms according to the NCI-CTG Expanded Common Toxicity Criteria (see Table 30) on Day 12.

Each individual will be assessed at the end of each treatment cycle for signs of adverse events and

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disease related signs and symptoms according to the NCI-CTG Expanded Common Toxicity Criteria (see Table 30).

All events related or not to the test article will be recorded in the CRF.

Table 30
NCIC-CTG Expanded Common Toxicity Criteria

TOXICITY	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
HAEMATOLOGICAL					
WBC x 10 ⁹ /l	≥ 4.0	3.0 - 3.9	2.0 - 2.9	1.0 - 1.9	< 1.0
Platelets x 10 ⁹ /l	WNL	75- normal	50 - 74	25 - 49	< 25
Hemoglobin g/ g/l	WNL WNL WNL	10.0 - normal 100 - normal 6.2 - normal	8.0 - 9.9 80 - 90 4.95 - 6.1	6.5 - 7.9 65 - 79 4.0 - 4.9	< 6.5 < 65 < 4.0
mmol/l					
Granulocytes/Bands x10 ⁹ /l	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
Lymphocytes x 10 ⁹ /l	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
Hematological – other	None	Mild	moderate	severe	life-threatening
HEMORRHAGE (clinical)	None	mild, no transfusion	gross, 1-2U per episode	gross, 3-4U per episode	massive, > 4U per episode
INFECTION	None	mild, no active treatment	moderate, PO antibiotic	severe, IV, antibiotic, antifungal or hospitalization	life-threatening
ALOPECIA	no loss	mild hair loss	pronounced or total head hair loss	total body hair loss	-
GASTROINTESTINAL					
Nausea	None	able to eat, reasonable intake	intake significantly decreased but can eat	no significant intake	-
Vomiting	None	once in 24 hrs	2-5x in 24 hrs	6-10x in 24 hrs	> 10x in 24 hrs or requiring IV support
Diarrhea	None	increase of 2-3 stools/day over pre Rx	increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	increase of 7-9 stools/day, or incontinence, or severe cramping	increase of ≥ 10 stools/day or grossly bloody diarrhea, or need for parenteral support
Stomatitis	None	painless ulcers, erythema, or mild soreness	painful erythema, oedema, or ulcers, but can eat	painful erythema, oedema, or ulcers, and cannot eat	requires parenteral or enteral support
Oesophagitis / dysphagia	None	painless ulcers, erythema, mild soreness or dysphagia	painful erythema, edema, or ulcers or moderate dysphagia but can eat without narcotics	cannot eat solids, or requires narcotics to eat	requires parenteral or enteral support or complete obstruction or perforation
Anorexia	None	Mild	moderate	severe	life-threatening

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TOXICITY	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Gastrointestinal (cont.)					
Gastric / Ulcer	No	Antacid	requires vigorous medical management or nonsurgical treatment	uncontrolled by medical management; requires surgery	perforation or bleeding
Small bowel obstruction	No	-	intermittent, no intervention	requires intervention	requires operation
Intestinal fistula	No	-	-	yes	-
Gastrointestinal – other	None	Mild	moderate	severe	life-threatening
Other Mucosal	None	erythema or mild pain not requiring treatment	patchy and serosanguinous discharge or nonnarcotic for pain	confluent fibrinous mucositis or ulceration or narcotic for pain	necrosis
LIVER					
Bilirubin	WNL	-	< 1.5 x N	1.5 - 3.0 x N	> 3.0 x N
Transaminase					
SGOT / AST	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
SGPT / ALT	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
AlkPhos or 5'nucleotidase	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
Liver – clinical	no change from baseline	-	-	precoma	hepatic coma
Liver – other	-	Mild	moderate	severe	life-threatening
Weight gain	< 5.0%	5.0 - 9.9%	10.0 - 19.9%	≥ 20%	-
Weight loss	< 5.0%	5.0 - 9.9%	10.0 - 19.9%	≥ 20%	-
RENAL AND BLADDER					
Creatinine	WNL	< 1.5 x N	1.5 - 3.0 x N	3.1 - 6.0 x N	> 6.0 x N
Proteinuria	no change	1+, or < 0.3g%, or < 3g/l	2-3+, or 0.3-1.0%:, or 3-10g/l	4+, or > 1.0g%, or > 10g/l	nephrotic syndrome
Hematuria	Neg	micro only	gross, no clots	gross + clots	requires transfusion
BUN mg% mmol/l	WNL, < 20 WNL, < 7.5	21 - 30 7.6 - 10.9	31 - 50 11.0 - 18.0	> 50 > 18.0	- -
Hemorrhagic cystitis	None	blood on microscopic examination	frank blood, no treatment required	bladder irrigation required	requires cystectomy or transfusion
Renal failure	-	-	-	-	dialysis required
Incontinence	Normal	with coughing, sneezing, etc.	spontaneous, some control	no control	- -

1. *Chrysomelidae* (1000)
 2. *Curculionidae* (1000)
 3. *Chrysomelidae* (1000)
 4. *Curculionidae* (1000)
 5. *Chrysomelidae* (1000)
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TOXICITY	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
DERMATOLOGICAL					
Skin	None or no change	scattered macular or papular eruption or eryhema that is asymptomatic	scattered macular or papular eruption or eryhema with pruritis or other associated symptoms	generalised symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Local	None	Pain	pain and swelling with inflammation or phlebitis	ulceration	plastic surgery indicated
CARDIAC					
Cardiac dysrhythmias	None	asymptomatic transient, no therapy required	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension or ventricular tachycardia or fibrillation
Cardiac function	None	asymptomatic, decline of resting LVEF \leq 20% of baseline	asymptomatic, decline or resting LVEF > 20% of baseline	mild CHF, responsive to therapy	severe or refractory CHF
Cardiac ischemia	None	non-specific T-wave flattening	asymtomatic, ST = T-wave changes for ischemia	angina without evidence for infarction	acute myocardial infarction
Cardiac pericardial	None	asymptomatic effusion, no intervention	pericarditis (rub, chest pain, ECG changes)	symptomatic effusion; drainage	drainage urgently required
Cardiac- other	-	Mild	moderate	severe	lfe-threatening
Hypertension	None or no change	asymptomatic, transient increase by > 20mmHg (D) or to > 150/100 if previously WNL; no treatment	recurrent or persistent increase by > 20mmHg (D) or to > 150/100 if previously WNL; no treatment	requires therapy	hypertensive crisis
Hypotension	None or no change	changes requiring no therapy (incl. transient orthostatic hypotension)	requires fluid replacement or other therapy but not hospitalization	requires therapy and hospitalization, resolves < 48 hrs of stopping the agent	requires therapy and hospitalization for > 48hrs. after stopping the agent
Phlebitis/thrombosis embolism	--	--	superficial phlebitis (not local)	deep vein thrombosis	major event (ceIECral/hepatic /pulmonary/ other infarction) or pulmonary embolism
Oedema	None	1 + or dependent in evening only	1 + or dependent in evening only	3+	4+, generalized diascara

TOXICITY	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
NEUROLOGICAL					
Neuro-sensory	none or no change	mild paresthesia; loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paresthesia	severe objective sensory loss or paresthesia that interfere with function	--
Neuro-motor	none or no change	Individualive weakness; no objective findings	mild objective weakness but no significant impairment of function	objective weakness with impairment of function	paralysis
Neuro- cortical	None	Mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation or hallucinations	coma, seizures, toxic psychosis
Neuro – ceIECellar	None	Slight incoordination, dysdiadochokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor ataxia	ceIECellar necrosis
Neuro – mood	no change	Mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuro – headache	None	Mild	moderate or severe but transient	unrelenting and severe	-
Neuro – constipation	none or no change	Mild	moderate	severe	ileus > 96 hours
Neuro – hearing	none or no change	Asymptomatic, hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro – vision	none or no change	--	--	symptomatic subtotal loss of vision	blindness
Pain	None	Mild	moderate	severe	intolerable
Behavioural change	None	Change, not disruptive to individual or family	disruptive to individual or family	harmful to others or self	psychotic behavior
Dizziness/vertigo	None	non-disabling	--	disabling	--
Taste	Normal	Slightly altered taste, metallic taste	markedly altered taste	--	--
Insomnia	Normal	Occasional difficulty sleeping, may need pills	--	difficulty sleeping despite medication	--
Neurological – other	--	Mild	moderate	severe	life-threatening

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TOXICITY	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
ALLERGY	None	transient rash, drug fever < 38°C.	urticaria, drug fever ≥ 38°C, mild bronchospasm	serum sickness, bronchospasm, parenteral meds	anaphylaxis
FLU-LIKE SYMPTOMS					
Fever in absence of infection	none	37.1 - 38.0°C	38.1 - 40.0°C	> 40.0°C < 24 hours	> 40.0°C > 24 hrs or fever with hypotension
Chills	None	mild or brief	pronounced or prolonged	-	-
Myalgia / Arthralgia	Normal	Mild	decrease in ability to move	disabled	-
Sweats	Normal	mild and occasional	frequent or drenching	-	-
Malaise	None	mild, able to continue normal activities	impaired normal daily activity or bedrest < 50% of waking hours	in bed or chair > 50% of waking hours	bedridden or unable to care for self
Flu-like symptoms – other	-	Mild	moderate	severe	life-threatening
METABOLIC					
Hyperglycemia mg/dl	< 116 < 6.2	116 – 160 6.2 – 8.9	161 - 250 9.0 - 13.9	251 - 500 14.0 - 27.8	> 500 > 27.8 or ketoacidosis
mmol/l					
Hypoglycemia mg/dl	> 64 > 3.6	55 – 64 3.1 – 3.6	40 - 54 2.2 - 3.0	30 - 39 1.7 - 2.1	< 30 < 1.7
mmol/l					
Amylase	WNL	< 1.6 x N	1.5 - 2.0 x N	2.1 - 5.0 x N	> 5.1 x N
Hypercalcemia mg/dl	< 10.6 < 2.66	10.6 - 11.5 2.65 - 2.87	11.6 - 12.5 2.88 - 3.12	12.6 - 13.5 3.13 - 3.37	> 13.5 > 3.37
mmol/l					
Hypomagnesemia mmol/l	> 0.70	0.70 - 0.58	0.57 - 0.38	0.37 - 0.30	≤ 0.29
Hyponatremia mmol/l	WNL or > 135	131 – 135	126 - 130	121 - 125	≤ 120
Hypokalemia mmol/l	WNL or > 3.6	3.1 – 3.6	2.6 - 3.0	2.6 - 3.0	≤ 2.0
Metabolic – other	- -	Mild	moderate	severe	life-threatening
COAGULATION					
Fibrinogen	WNL	0.99 - 0.75 x N	0.74 - 0.50 x N	0.49 - 0.25 x N	≤ 0.24 x N
Prothrombin time	WNL	1.01 - 1.25 x N	1.26 - 1.50 x N	1.51 - 2.00 x N	> 2.00 x N
Partial thromboplastin time	WNL	1.01 - 1.66 x N	1.67 - 2.33 x N	2.34 - 3.00 x N	> 3.00 x N
Coagulation – other	- -	Mild	moderate	severe	life-threatening

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TOXICITY	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
EYE					
Conjunctivitis / keratitis	None	none erythema or chemosis, no steroids or antibiotics	steroids or antibiotics required	corneal ulceration or visible opacification	-
Dry eye	Normal	-	requires artificial tears	-	requires enucleation
Glaucoma	no change	-	-	yes	-
Eye – other	-	Mild	moderate	severe	life-threatening
ENDOCRINE					
Impotence / libido	Normal	decrease in normal function	--	absence of function	--
Sterility	--	--	yes	--	--
Amenorrhea	No	Yes	--	--	--
Gynaecomastia	Normal	Mild	pronounced or painful	--	--
Hot flushes	None	mild or <1/day	moderate and ≥1/day	frequent and interferes with normal function	--
Cushingoid	Normal	Mild	pronounced	--	--
Endocrine – other	--	Mild	moderate	severe	life-threatening
OTHER	--	Mild	moderate	severe	life-threatening

WNL= within normal limits

5 Safety Laboratory Determinations

All laboratory tests with values that become abnormal to a clinically important degree after test article administration should be repeated until the values return to normal or baseline. If laboratory values do not return to normal or baseline within a reasonable period, the etiology should be identified and the sponsor notified.

STATISTICAL ANALYSIS

Statistical analyses will be performed by the Biostatistical Section of the sponsor; any additional or supplemental analyses performed independently by the investigator should be submitted to the sponsor.

Statistical and Analytical Plans

Efficacy analysis

The analyses of efficacy endpoints will be performed with both the intent-to-treat and per-protocol populations. The analyses of safety endpoints will be performed with the safety population.

The primary efficacy variables are the overall response rate and time to disease progression. To evaluate the overall response rate, two sets of analyses will be performed, one on the intent-to-treat approach (all individuals) and one based on data from evaluable individuals only, meaning all eligible individuals who complete at least two cycles of therapy and undergo tumor evaluation.

The analysis of the time to disease progression will include all eligible individuals who have received at least one treatment cycle of test article. Time to disease progression is defined as time from beginning of treatment until documented disease progression (PD). An individual who died for any reason will be considered as PD and the date of progression will be the date of death unless PD is documented before death.

Duration of overall response and individual's survival are the secondary efficacy variables. Duration of response will be assessed on responder individuals only and is defined as the time from the measurement criteria is met for Complete Response (CR) or Partial Response (PR) (which ever status is recorded first) until the first date that PD is objectively documented.

All individuals will be followed for survival, which will be measured from the beginning of the treatment therapy until death.

Safety analysis

The analysis of the safety will be based on the Safety population (SAF). No inferential statistics will be conducted on the safety variables.

The adverse events will be coded using standard dictionaries and their incidences will be described per preferred term and treatment, overall and regarding their severity and their relationship with the test article.

The same analysis will be carried out for all grade 3 / 4 toxicities.

Evaluation Criteria

All individuals who were not enrolled or who did not received at least one treatment cycle of test article will be excluded from the intent-to-treat population (ITT). The per-protocol population (PPS) will be a subset of the ITT population with individuals who are meeting all inclusion and exclusion criteria and are evaluable for the objective tumor response.

All individuals who were not enrolled or who did not received at least one dose of test article will be excluded from the safety analysis.

5 CONTRAINDICATIONS, PRECAUTIONS, AND WARNINGS

For a complete description of all AEs reported during amonafide clinical studies, please refer to the amonafide Investigator's Brochure provided.

10 ADVERSE EVENTS

Definitions

The term "adverse event", as used by the sponsor is synonymous of the term "adverse experience".

15 An **adverse event** is any untoward, undesirable, unplanned clinical event in the form of signs, symptoms, or laboratory or physiological observations occurring in a human being participating in a clinical study with a sponsor test article regardless of a causal relationship.

20 This includes the following:

- 1) Any clinically significant worsening of a preexisting condition;
- 2) Any reoccurrence of a preexisting condition;
- 25 3) An AE occurring from overdose of a sponsor test article whether accidental or intentional (meaning, a dose higher than that prescribed by a health care professional for clinical reasons);

2025-09-26 09:00

- 4) An AE occurring from abuse of a sponsor test article (meaning, use for non clinical reasons); or
- 5) An AE that has been associated with the discontinuation of the use of a sponsor test article.

Note: A procedure is not an AE, but the reason for a procedure may be an AE.

10

A **preexisting condition** is a clinical condition (including a condition being treated) that is diagnosed before the individual signs the informed consent and is documented as part of the individual's medical history.

15

The questions concerning whether the condition existed before the start of the active phase of the study and whether it has increased in severity and/or in frequency will be used to determine whether an event is a treatment-emergent adverse event (TEAE). An AE is considered to be treatment emergent if: (1) it was not present when the active phase of the study began and is not a chronic condition that is part of the individual's medical history; or (2) it was present at the start of the active phase of the study or as part of the individual's medical history, but the severity or frequency increased during the active phase.

25

The active phase of the study begins at the time of the first dose of test article.

2025-09-26 10:00:00

According to ICH guidelines, a **serious adverse event** is any AE occurring at any dose that meets one or more of the following criteria:

- 5 1) Results in death (occurring within **30 days** after the last test article administration);
- 2) Is life-threatening (see below);
- 3) Requires inindividual hospitalization or prolongation of an existing hospitalization
- 10 (see below);
- 4) Results in persistent or significant disability or incapacity (see below);
- 5) Results in secondary cancer; or
- 6) Results in a congenital anomaly or birth
- 15 defect.

Additionally, important events that may not result in death, be life-threatening, or require hospitalization may be considered SAE when, based on appropriate medical

20 judgment, they may jeopardize the individual and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such events included allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood

25 dyscrasias or convulsions that do not require hospitalization, or development of drug dependency or drug abuse.

A **life-threatening adverse event** is any AE that places the individual at immediate risk of death from the

30 event as it occurred; a life-threatening event does not

include an event that, had it occurred in a more severe form, might have caused death, but as it actually occurred, did not create an immediate risk of death. For example, drug-induced hepatitis that resolved without
5 evidence of hepatic failure would not be considered life-threatening, even though drug-induced hepatitis of a more severe nature can be fatal.

Hospitalization is to be considered only as an overnight admission. Hospitalization or prolongation of a
10 hospitalization constitutes criteria for an AE to be serious. In the absence of an AE, there should not be any reporting for a hospitalization or prolongation of a hospitalization by the participating investigator through a Serious Adverse Event Form (Form SAE 4500). This is the
15 case in the following situations:

- 1) The hospitalization or prolongation of hospitalization is needed for a procedure required by the protocol;
- 20 2) The hospitalization or prolongation of hospitalization is part of a routine procedure followed by the center (e.g., stent removal after surgery). This should be recorded in the study file;
- 25 3) The hospitalization for survey visits or annual physicals fall in the same category.

In addition, hospitalizations planned before the start of the study, for a preexisting condition that has
30 not worsened do not constitute an SAE (e.g., elective

hospitalization for a total knee replacement due to a preexisting condition of osteoarthritis of the knee that has not worsened during the course of the study).

Disability is defined as a substantial disruption
5 in a person's ability to conduct normal life functions.

If there is any doubt whether the information constitutes an AE or SAE, the information is treated as an SAE.

10 Reportable Events/Information

AEs reportable to the sponsor include the following:

All SAEs and AEs, the onset of which occur during the course of the study with the sponsor test article
15 (study drug, placebo, or control drug agent), since signing the informed consent form until 15 days (AEs) or 30 days (SAEs) from the individual's last dose, regardless of test article or protocol relationship.

All non serious AEs, the onset of which occur
20 during the course of the study with the sponsor test article, since signing the informed consent form, including the screening, and within the established follow-up period for safety as required by the protocol.

The following events will be recorded and reported
25 in the same time frame and using the same process as SAEs:

All pregnancies diagnosed during the course of the study and their outcome. If a individual is confirmed to be pregnant during the study, the continued use of the test article must be evaluated immediately.
30 Administration of the test article should be discontinued

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unless the potential benefit to the individual justifies the potential risk to the fetus. The use of an approved drug may be discontinued if no evidence has been revealed (e.g., in the package insert) that it has teratogenic effects. All reports of pregnancy must be followed for information about the course of the pregnancy and delivery, as well as the condition of the newborn. The investigator will provide follow-up information regarding the outcome of the pregnancy to the sponsor monitor in a timely manner. This information will be provided regardless of whether the individual has discontinued participation in the study. When the newborn is healthy, additional follow-up is not needed.

Test article abuse and overdose (i.e., use for non clinical reasons) with or without AEs. Overdoses means that the individual was prescribed or has taken a dose higher than the dose provided in the protocol. It is up to the participating investigator to decide whether a dose was an overdose.

Inadvertent or accidental exposure to test article with or without an AE: This includes post-study test article-related SAEs; SAEs occurring after an unauthorized or accidental use in persons who do not participate in the study; and abnormal biological or vital sign values that are considered clinically relevant by the participating investigator. These must be reported in the same time frame and following the same process as an AE or an SAE.

Recording and Reporting

At each required visit during the study, all AEs that have occurred since the previous visit must be

recorded in the adverse event record of the individual's CRF. The information recorded should be based on the signs or symptoms detected during the physical examination and clinical evaluation of the individual. In addition to
5 the information obtained from those sources, the individual should be asked the following nonspecific question: "How have you been feeling since your last visit?" Signs and symptoms should be recorded using standard medical terminology.

10 The following information must be included (when applicable): the specific condition or event and direction of change; whether the condition was preexisting (i.e., an acute condition present at the start of the study or history of a chronic condition) and, if so,
15 whether it has worsened (e.g., severity and/or frequency); the dates of occurrence; severity, causal relationship to test article; action(s) taken; and outcome. This includes AEs that occur during screening period. The collection of AE data should continue for 15 days after test article
20 discontinuation. (For SAEs, see below.)

The causal relation between an AE and the test article will be determined by the investigator on the basis of his or her clinical judgment and the following definitions:

25 **Definitely related:** Event can be fully explained by administration of the test article;

Probably related: Event is most likely to be explained by administration of the test article, rather than the individual's clinical state or other
30 agents/therapies;

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Possibly related: Event may be explained by administration of the test article, or by the individual's clinical state or other agents/therapies;

Probably not related: Event is most likely to be explained by the individual's clinical state or other agents/therapies;

Definitely not related: Event can be fully explained by the individual's clinical state or other agents/therapies.

10

When assessing the relationship between administration of the test article and the AE, the following should be considered:

- 15 1) Temporal relationship between administration of the test article and the AE;
- 2) Biological plausibility of relationship;
- 3) Individual's underlying clinical state or concomitant agents and/or therapies;
- 20 4) Where applicable, whether the AE abates on discontinuation of the test article (dechallenge);
- 5) Where applicable, whether the AE reappears on repeat exposure to the test article
- 25 (rechallenge).

SAEs from clinical studies that are not test article-related may nevertheless be considered by the participating investigator or medical monitor (or
30 designee) to be related to the conduct of the protocol,

i.e. related to the fact that a individual is participating in the study. For example, a protocol-related SAE may be an event occurring during the washout period or related to a procedure required by the protocol.

5 The severity of AEs will be assessed according to the NCIC Expanded Common Toxicity Criteria (Table 30). The following definitions should be used for toxicities that are not defined in the NCIC Expanded Common Toxicity Criteria:

10

1) Mild (Grade 1): The AE is noticeable to the individual but does not interfere with routine activity;

15

2) Moderate (Grade 2): The AE interferes with routine activity but responds to symptomatic therapy or rest;

3) Severe (Grade 3): The AE significantly limits the individual's ability to perform routine activities despite symptomatic therapy;

20

4) Life-threatening (Grade 4): The individual is at immediate risk of death.

25 All documentation pertaining to the event (e.g., additional laboratory tests, consultation reports, discharge summaries, postmortem reports, etc.) will be provided by the investigator to the sponsor monitor in a timely manner.

 Reports relative to the individual's subsequent course must be submitted to the sponsor until the event

has subsided or, in case of permanent impairment, until the condition stabilizes.

Any emergency must be reported to the sponsor medical monitor (or designee) immediately (within 24
5 hours) by contacting the medical monitor listed in the front of this protocol.

Any SAE, regardless of causal relationship, must be reported to a sponsor medical (or designee) immediately (within 24 hours) by faxing a completed Serious Adverse
10 Event Form (Form SAE 4500) to the number indicated in the front of this protocol and confirming that the fax was received. This must be done so that the sponsor may comply with its regulatory obligations.

The individual should be observed and monitored
15 carefully until the condition resolves, stabilizes, or its cause is identified. Follow-up information relating to an SAE must be reported to the sponsor medical (or designee) immediately (within 24 hours) by faxing a completed Serious Adverse Event Form (Form SAE 4500) to the number
20 indicated in the front of this protocol and confirming that the fax was received as soon as additional data related to the event are available.

Death may occur as a result of the disease. Nevertheless, all deaths which occur within 30 days of the
25 last administration of test article, or which may be considered as related to the test article agent, regardless of the interval, must be treated as a SAE and reported as such.

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The investigator is responsible for reporting adverse events to his/her Ethics Committee (EC), according to local regulations.

In agreeing to the provisions of this protocol,
5 these responsibilities are accepted by the investigator.

For all other inquiries and information about this study, contact a study manager listed in the front of this protocol.

10 DATA QUALITY ASSURANCE

The sponsor performs quality control and assurance checks on all clinical studies that it sponsors. Before enrolling any individual into this study, sponsor personnel and the investigator will review the protocol,
15 the brochure for clinical investigators, the CRFs and instructions for their completion, the procedure for obtaining informed consent, and the procedure for reporting AEs and SAEs. A qualified representative of the sponsor will monitor the conduct of the study by visiting
20 the site and by contacting the site by telephone. During the visits, information recorded on the CRFs will be verified against source documents. After the sponsor receives the CRFs, the sponsor medical monitor will review the forms for safety information, and the sponsor clinical
25 data associates will review them for legibility, completeness, and logical consistency. The data will be entered into a database using a double entry procedure. Additionally, the sponsor clinical data associates will use automated validation programs to help identify missing
30 data, selected protocol violations, out-of-range data, and

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other inconsistencies. Requests for data clarification or correction will be forwarded to the investigative site for resolution.

5 INVESTIGATOR'S REGULATORY OBLIGATIONS

Ethics Committee (EC) Approval

The protocol and the informed consent document must have the initial and at least annual approval of an
10 EC. The signed EC approval letter must identify the documents approved (i.e. list the investigator's name, the protocol number and title, the date of the protocol and informed consent document, and the date of approval of the protocol and the informed consent document). Any
15 advertisement used to recruit individuals also should be reviewed by the EC. The sponsor will not ship clinical supplies until a signed approval letter from the EC has been received and a contractual agreement has been signed by the sponsor and the clinical site. Copies of the
20 regulations relating to ethics committees are available from the sponsor.

Study Documentation

The investigator must provide the sponsor with the
25 following documents BEFORE the enrollment of any individuals:

Completed and signed FDA Form 1572 (Statement of Investigator);

30 All applicable country specific regulatory forms;

Current, signed curricula vitae of the investigator and all sub-investigators;

Copy of the EC approval letter for the protocol and informed consent. Written assurance of continuing approval (at least annually) as well as a copy of the annual progress report submitted to the EC must also be provided to the sponsor. Any changes in this study or unanticipated problems involving risks to the individuals must be reported promptly to the EC. An investigator must not make any changes in a study without EC and sponsor approval except when necessary to eliminate apparent immediate hazards to the individuals. All protocol amendments must be submitted to the EC and approved;

Copy of the EC approved informed consent document to be used;

Written documentation of EC approval of recruitment advertising (if applicable);

When applicable, a list of EC members and their qualifications, and a description of the committee's working procedures;

Protocol Approval Page signed by the investigator;
Fully executed Clinical Trial Agreement;

A written document containing the name, location, certification number, and date of certification of the laboratory to be used for laboratory assays and those of other facilities conducting tests. The sponsor must be notified if the laboratory is changed or if any additional laboratory is to be used;

List of normal laboratory values and units of measurements for all laboratory tests required by the

protocol. This is required for each laboratory to be used during the study. The sponsor must be notified if normal values or units of measurement change.

5 During the study, the investigator must maintain in order the following administrative documents related to the study:

- 10 1) Photocopy of the signed Protocol Approval Page;
- 2) Curricula vitae of all personnel involved in the study;
- 3) Photocopy of the signed FDA Form 1572;
- 15 4) EC Approval Notification for:
- a) Protocol;
- b) Informed consent document;
- c) Recruitment advertising (if applicable);
- d) Amendment(s) (if applicable);
- e) Annual review of the protocol and the
- 20 informed f) consent document;
- g) Serious Adverse Events;
- h) Study closure.
- 5) Reports of Serious Adverse Events;
- 6) Drug Inventory Forms (drug receipts, drug
- 25 dispensing and inventory forms);
- 7) Name and address of local laboratory, list of normal laboratory values and units of measurements, as well as laboratory certification or hospital accreditation;

- 8) Correspondence with the sponsor.

Informed consent

Regulatory agencies have issued regulations to
5 provide protection for human individuals in clinical
investigations and describe the general requirements for
informed consent.

A copy of your proposed informed consent document
should be submitted to the sponsor for review and comment
10 before submission to your EC. The study should not begin
until the document has been reviewed by the sponsor and
until the document has been approved by the EC. In some
instances the study must not begin until the document has
been approved by a regulatory agency.

The informed consent document shall contain all
15 the elements of informed consent specified in the
regulations. Some regulations may require the disclosure
of additional information to the individual and/or
inclusion of additional information in an informed consent
20 document. Copies of the regulations relating to informed
consent and the protection of human individuals in
clinical studies are available from the sponsor.

Nothing in these regulations is intended to limit
the authority of a physician to provide emergency medical
25 care under applicable regulations. In addition, you
should be aware that some regulations require that you
permit regulatory agencies to conduct inspections and
review records pertaining to this clinical investigation.

2025-09-26 10:00:00

Declaration of Helsinki

This study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, and that are consistent with GCP
5 and the applicable regulatory requirements.

Case Report Form

All data (laboratory data and investigator/study personnel observations) will be recorded on CRFs provided
10 by the sponsor. ECG, chest X-rays and CT scans must be reported in summary in the CRF. The original reports, traces and films must be retained by the investigators for future reference.

The CRFs must be completed in black ink and
15 corrections made by striking out any errors, with a single stroke, and not by using correction fluid, which is prohibited by the regulations governing pharmaceutical trials. All corrections must be initialed and dated.

Original CRFs will be collected at regular
20 intervals by the sponsor monitor and copies of the CRFs must be retained by the investigators, for as long as is legally required after completion of the study.

CRFs and other pertinent records are to be submitted to the sponsor during and/or at termination or
25 completion of the study.

The investigator also must submit all incomplete CRFs that document individual experience with the test article, including retrievable data on individuals who withdraw before completion of the study.

Adverse Event Reporting

The investigator agrees to report all AEs to the sponsor as described in the Adverse Event section.

Furthermore, the investigator is responsible for ensuring
5 that any sub-investigator promptly brings AEs to the attention of the investigator. If applicable, the investigator is also responsible for informing the participating EC of any SAEs.

10 Review of Source Records

The investigator agrees that qualified representatives of the sponsor and regulatory agencies will have the right, both during and after this study, to conduct inspections, and to audit and review medical
15 records pertinent to the clinical study as permitted by regulations. Individuals will not be identified by name, and confidentiality of information in medical records will be preserved. The confidentiality of the individual will be maintained unless disclosure is required by
20 regulations. Accordingly, the following statement (or similar statement) that permits the release of the individual's medical records will be included in the informed consent document:

25 Monitoring of the Study

The study will be monitored by a representative of the sponsor. Site visits will be made before the study begins, at regular intervals during the study, and at the study closeout. The frequency of the monitoring visits
30 will depend on the rate of enrollment, time frame between

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individual visits, number of individuals enrolled in the study and the data entry cut-off dates. As part of regular monitoring visits, the sponsor monitor will check 100% of available source documents against the CRFs.

- 5 Communication by telephone and mail and e-mail may be used as needed to supplement site visits. The investigator and study personnel will cooperate with the sponsor, provide all appropriate documentation, and be available to discuss the study. The purpose of the site visits is to verify:

10

Adherence to the protocol (The investigator should document and explain any deviations from the approved protocol);

- 15 The completeness and accuracy of completion of the CRFs and the dispensing and inventory record (Adequate time and space for these visits should be allocated by the investigator);

- 20 Compliance with the regulations. The verification will require comparison of the source documents against the CRFs.

Amendments to the Protocol

- Any significant change to the study protocol will result in an amendment. The investigator and the appropriate sponsor medical monitor will indicate their approval by signing the approval page of the amendment. Once a protocol amendment has received approval from the sponsor, the investigator will submit it to the EC for written approval. The approval letter, signed by the EC chairman, must refer specifically to the investigator, the
- 25
- 30

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5 sponsor protocol number and title, the protocol amendment number and the date of the protocol amendment. The sponsor will submit a copy of the protocol amendment to the appropriate regulatory agency/agencies. A protocol amendment may be implemented after it has been approved by the EC.

10 In case of a protocol change intended to eliminate an apparent immediate hazard to individuals, the change may be implemented immediately, but the change must then be documented in an amendment and reported to the EC within 5 working days.

Change in Investigator

15 If any investigator retires, relocates, or otherwise withdraws from conducting a study, the responsibility for maintaining records may be transferred to another person (sponsor, EC, other investigators) who will accept the responsibility. The sponsor must be notified of and agree to the change. An updated FDA Form 1572 will be filed with the sponsor and the FDA for any changes in the study personnel reported in the current FDA Form 1572.

25 TERMINATION OF STUDY

Sponsor Termination

30 The sponsor may terminate the study at any time for any of the following reasons:

- 1) Failure to enroll individuals;
2) Protocol violations;
3) Inaccurate or incomplete data;
5 4) Unsafe or unethical practice;
5) Questionable safety of the test article;
6) Suspected lack of efficacy of the test
article;
7) Administrative decision.

10

Investigator Termination

15 If the investigator terminates the study
prematurely, the investigator will do the following:

Return all test articles, CRFs, and related study
materials to the sponsor;

20 Provide a written statement describing why the
study was terminated prematurely.

Final Study Report

The investigator will complete a report notifying
25 the EC of the conclusion of the clinical study. This
report should be made within 3 months of completion or
termination of the study.

The final report sent to the EC will also be sent
to the sponsor and, along with the completed CRFs, will

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constitute the final summary to the sponsor, thereby fulfilling the investigator's regulatory responsibility.

Confidentiality

5 All unpublished information given to the investigator by the sponsor shall be kept confidential and shall not be published or disclosed to a third party without the prior written consent of the sponsor.

10 When the sponsor generates reports for presentations to regulatory agencies, an endorsement of the final report will be sought from one or more of the investigators who have contributed significantly to the study. The endorsement is required by some regulatory agencies.

15 No patent application based on the results of this study shall be made by the investigator, and no assistance given to any third party for such an application, without the written authorization of the sponsor.

20 Records Retention

All correspondence related to this clinical study should be kept in appropriate study files. Records of individuals, source documents, CRFs, drug inventory, and EC and sponsor correspondence pertaining to the study must
25 be kept on file. All original individual, laboratory, and drug inventory records relating to the study should be retained for not less than two (2) years after the last approval of a marketing application in an ICH region and until there are no pending or contemplated marketing
30 applications in an ICH region or at least two (2) years

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have elapsed since the formal discontinuation of clinical development of the investigational product. These documents should be retained for a longer period, however, if required by applicable regulatory requirements or by an agreement with the sponsor. Thereafter, records will not be destroyed without giving the sponsor prior written notice and the opportunity to further store such records.

Publications

On completion of the study, the investigator may publish the results in recognized (refereed) scientific journals if the data warrant publication. The sponsor requires that manuscripts based on this study receive its approval before submission. This is to safeguard against disclosure of confidential information provided by the sponsor. A draft manuscript must be submitted to the sponsor before submission to the journal. Abstract of papers to be presented also must be forwarded to the sponsor at least 30 days before they are submitted for consideration for presentation. Proposed publication and abstract will be reviewed promptly.